

Dissertation

submitted to the

Combined Faculties for the Natural Sciences and for Mathematics

of the Ruperto-Carola University of Heidelberg, Germany

for the degree of

Doctor of Natural Sciences

presented by

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Oral-examination: 10.03.2016

**Loss of tumorigenicity by
transdifferentiation:
from squamous cell carcinoma to
melanocyte-like cells**

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Declarations according to § 8 (3) b) and c) of the doctoral degree regulations:

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Name

*This thesis is dedicated to my parents
who gave me roots and wings.*

Parts of this thesis have been published in:

Fehrenbach, S., Novak, D., Utikal, J.

Poster presentation: 'Transdifferentiation of cancer cells –from the keratinocytic lineage into the melanocytic lineage'

DKFZ PhD Retreat, July 2014, Weil der Stadt, Germany

Fehrenbach, S., Novak, D., Utikal, J.

Poster presentation: 'Change Identity –take the direct way: from the keratinocytic lineage into the melanocytic lineage'

9th International PhD Student Cancer Conference, June 2015, Christie NHS Foundation Trust Manchester, England

Fehrenbach, S., Novak, D., Utikal, J.

Poster presentation: 'Change Identity –take the direct way: from the keratinocytic lineage into the melanocytic lineage'

World Conference on Regenerative Medicine, October 2015, Leipzig, Germany

Fehrenbach, S., Novak, D., Utikal, J.

Poster presentation: 'Loss of tumorigenicity by direct conversion'

PhD Poster presentation November 2015, DKFZ, Heidelberg, German

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III. Abstract

Lineage-specific transcription factors determine the cell fate during development. Remarkably, several studies have demonstrated that ectopic overexpression of specific transcription factors can promote the direct conversion of one somatic cell type into another. Even the lineage of origin of cancer cells could be switched by this mechanism and the resulting cells showed impaired tumor forming potential. This approach might therefore be suitable as an alternative therapeutical strategy for the treatment of cancer. The aim of this project was to directly convert tumor cells from the keratinocytic lineage into functional and potentially non-tumorigenic cells from the melanocytic lineage. We hypothesize that the tumorigenic potential of a cancer cell is dependent on the differentiation lineage. Thus, transdifferentiation of squamous cell carcinoma cells (SCCs) into the melanocytic lineage should yield non-tumorigenic cells.

In order to achieve this objective, different sets of candidate transcription factors were cloned into doxycycline-inducible lentiviral vectors and were ectopically overexpressed in a SCC line.

This thesis is a proof of principle that transdifferentiation of cancer cells is possible and that this process can lead to loss of tumorigenic potential. After ectopic overexpression of the four transcription factors MITF, SOX10, SOX9 and LEF1, morphological changes towards a melanocyte-like appearance could be observed. Moreover, keratinocyte markers were downregulated and melanocyte marker expression was induced. Additionally, melanosome-like structures were present in the transdifferentiated cells. On genomic level only minor changes were observed using array CGH. Also the global gene expression and the methylation landscape remained very similar between the parental SCC and the transdifferentiated cells. Yet, several characteristics including proliferation, cell metabolism, migration and invasion were reduced. Additionally the cells were less responsive to a cancer cell-specific drug combination using autophagy inhibition and an AKT inhibitor as well as to the treatment with the chemotherapeutic drug cisplatin. Finally, the transdifferentiated cells lost their tumorigenic potential, which could be shown *in vivo* by subcutaneous injection into immunosuppressed mice. To find a mechanism that could explain these observations, whole genome microarray and methylation array analyses were performed. After a gene set enrichment analysis (GSEA) IL-24 was identified as the top hit. Probably due to the transdifferentiation into the melanocytic lineage IL-24 became upregulated and might have affected the tumorigenic potential of the cells. In conclusion, we could identify a specific transcription factor combination which promotes the conversion of tumorigenic SCCs into nontumorigenic melanocyte-like cells.

IV. Zusammenfassung

Zelltypspezifische Transkriptionsfaktoren bestimmen die Differenzierungsrichtung einer Zelle während der Entwicklung. Mehrere Studien haben gezeigt, dass ektopische Überexpression von Transkriptionsfaktoren die direkte Umwandlung von einem somatischen Zelltyp in einen anderen fördern kann. Sogar die Differenzierungslinie von Krebszellen kann durch diesen Mechanismus verändert werden. Die daraus resultierenden Zellen hatten ein gemindertes Potenzial einen Tumor zu bilden. Auf Grund dieser Tatsache könnte sich diese Herangehensweise als therapeutische Alternative für die Behandlung von Krebserkrankungen erweisen. Das Ziel dieser Studie war es, Krebszellen der keratinozytären Linie in potentiell nicht-tumorigene Zellen der melanozytären Linie umzuwandeln. Unserer Hypothese zufolge ist das Potential einen Tumor zu formen von der Differenzierungslinie abhängig. Demnach sollten durch die Transdifferenzierung von Plattenepithelkarzinomzellen in melanozytäre Zellen nicht-tumorformende Zellen entstehen. Um dieses Ziel zu erreichen wurden verschiedene Transkriptionsfaktoren in doxycycline-induzierbare lentivirale Vektoren kloniert und daraufhin ektopisch in Plattenepithelkarzinomzellen überexprimiert. Diese Arbeit zeigt, dass die Transdifferenzierung von Krebszellen möglich ist. Zudem führt diese Umwandlung zum Verlust des tumorigenen Potentials. Nach der ektopischen Überexpression der vier Transkriptionsfaktoren MITF, SOX10, SOX9 und LEF1 konnten morphologische Veränderungen in Richtung des melanozytären Erscheinungsbilds beobachtet werden. Die Expression von Keratinozytenmarkern wurde runterreguliert und Melanozytenmarker wurden exprimiert und melanosomartige Strukturen waren in den transdifferenzierten Zellen vorhanden. Nur kleine Veränderungen auf der genomischen Ebene wurden bemerkt. Auch die globale Genexpression sowie der Methylierungsstatus zeigten kaum Veränderungen zwischen den ursprünglichen und den transdifferenzierten Zellen. Jedoch waren mehrere Eigenschaften wie Proliferation, Zellmetabolismus, Migration und Invasion deutlich reduziert. Zusätzlich waren die transdifferenzierten Zellen weniger sensitiv gegenüber Autophagie und AKT Inhibition sowie gegenüber der Behandlung mit dem Chemotherapeutikum Cisplatin. Letztendlich hatten diese Zellen ihr tumorformendes Potential verloren was *in vivo* mit der subkutanen Injektion in immunsupprimierte Mäuse gezeigt wurde. Eine Genset Anreicherungsanalyse von Genexpressionsanalyse sowie eine Methylierungsanalyse um einen Mechanismus zu identifizieren resultierte in IL-24 als Topkandidat. Wahrscheinlich wurde die Expression von IL-24 durch die Transdifferenzierung in die melanozytäre Richtung aktiviert und hat auch das tumorbildende Potential der Zellen beeinflusst. Zusammenfassend haben wir eine spezielle Kombination von Transkriptionsfaktoren identifiziert, die Plattenepithelkarzinomzellen in nicht-tumorigene melanozyten-ähnliche Zellen umwandeln kann.

V. Introduction

V.1. Structure and function of the skin

The skin constitutes the main barrier, which protects the body against pathogens and other harmful, environmental influences as well as dehydration. Further, the skin is involved in thermoregulation and sensation of touch. In adults, it accounts for about 16% of the total body weight and is the largest organ of the body [1, 2]. Two layers execute the functions of the skin: the epidermis and the dermis. The epidermis is the outer layer of the skin which is replaced by new cells within 6-8 weeks [3] and is subdivided into four major layers: the stratum basale, stratum spinosum, the stratum granulosum and the stratum corneum. The basal layer of the epidermis, known as the stratum basale, consists of proliferating and non-proliferating keratinocytes. These cells migrate outwards and differentiate on their way to form the other layers of the skin [4]. Attachment to the basement membrane is given by hemidesmosomes [5]. Besides keratinocytes, also melanocytes and Merkel cells are present in this layer. Melanocytes make contacts with the surrounding keratinocytes via their dendritic processes. In the middle of the following layer, the stratum spinosum, Langerhans cells, which are immunologically active cells, can be found [6]. In the stratum granulosum keratinocytes lose their nuclei and show a granular cytoplasm. The stratum corneum, the outermost layer, serves as protection against the environment. The stratum corneum reaches its greatest thickness on the palms of the hands and the soles of the feet. A fifth layer, the stratum lucidum which is a transition between stratum granulosum and stratum corneum, can only be found on the palms of the hands and soles of the feet [7, 8]. The main function of the dermis is to offer a supporting structure to the skin. Fibroblasts in the dermis produce collagen and elastin, which confer stability and elasticity. The dermis is well vascularized to supply the skin with nutrients. Additionally the temperature of the skin is regulated via the blood vessels and sweat glands in the dermis. At the dermo-epidermal junction nerve endings are present which sense touch, temperature, pain, and pressure [2, 9]. The hypodermis is the tissue below the dermis. Its function is to connect the skin to the underlying tissues. It consists mostly of fibroblasts and adipocytes, which store fat and serve as energy reserve, and is attached to the dermis by collagen and elastin fibers [9, 10].

V.2. Cell types in the skin

Different cell types are present in the skin serving various specific functions. Besides keratinocytes, melanocytes, Merkel cells, Langerhans cells, and fibroblasts build up the skin [1, 4].

Keratinocytes are the main cell type of the skin accounting for ~90% of the cells in the epidermis [6]. The skin layers are continuously renewed by active proliferation of keratinocytes. Starting at the basal layer keratinocytes travel across the epidermis to the stratum corneum. The cells in the stratum basale, which is a single layer of keratinocytes, divide and give rise to two daughter cells. One of the daughter cells remains in the basal layer while the other one migrates upwards and differentiates [2]. The main function of these cells is to produce the fibrous protein keratin, which fills their cytoplasm. On the way to the outer surface of the skin cells flatten and their nucleus becomes degenerated. Cohesion is increased by secretion of lipids, such as cholesterol, free saturated fatty acids and ceramides, hereby creating an efficient barrier [11]. Terminal differentiated keratinocytes in the outer layer, the stratum corneum, are called corneocytes. These cells have lost their nuclei, are completely flattened and filled with keratin which is necessary for the protective function of the skin [2]. A process called desquamation marks the end of the keratinocytic life cycle. Specific enzymes degrade cell-cell contacts leading to separation of the single corneocytes and ultimately loss of attachment at the surface of the skin [12].

About 6% of the cells in the skin are Merkel cells. These tactile cells are in contact with sensory nerve endings to sense different stimuli such as pressure thus functioning as mechanoreceptors. Merkel cells can be found alone or clustered together in so called Merkel corpuscles [13, 14].

Langerhans cells, a subset of dendritic cells were discovered by Paul Langerhans in 1868 and first thought to be nerve cells [15]. Yet, it is known that Langerhans cells are derived from the bone marrow and belong to the innate immune system. Due to their antigen-presenting ability they are also known as the 'Guardians of the skin' [16]. Approximately 3-8% of the cells in the epidermis are Langerhans cells [17].

Melanocytes are pigment-producing cells which are derived from the neural crest. These cells are responsible for the pigmentation of the skin and the hair [18]. Classical melanocytes reside in the basal layer of the epidermis or in the hair follicle and make up about 8% of cells in the skin [2]. Upon ultraviolet (UV) light exposure melanocytes transfer the pigment melanin to the surrounding keratinocytes leading to UV protection and preventing DNA damage [9, 19]. Melanocytes cannot only be found in the skin but are also present for example in the inner ear, the brain, the bones, and the heart [20].

The majority of cells in the dermis are fibroblasts, which reside mostly in the dermal papillae in proximity to the epidermis. The main function is to produce fibers of the extracellular matrix. Collagen provides the dermis with resistance to stress while it is responsible for the elasticity of the skin [9].

V.3. Melanocyte development

Melanocytes in the skin derive from neural crest (NC) stem cells. Several differentiation stages are necessary for generation of mature melanocytes. These stages can be distinguished by the location of the cells and distinct marker expression. The NC precursor cells are present in the truncal region between neural ectoderm and non-neural ectoderm. The fusion of the dorsal part of the neural tube leads to emergence of neural crest cells (NCC). These cells are now already specified and express the markers WNT1, paired box 3 (PAX3) and forkhead box D3 (FOXD3). Thereupon, cells undergo an epithelial to mesenchymal transition (EMT) before migrating into the migration staging area (MSA). The multipotent premigratory NCCs express the NC markers SOX9 (sex determining region Y-related HMG box 9) and SOX10 at this stage [18]. The NC gives rise to a variety of different cell types. During migration either along the dorsoventral or the dorsolateral pathway NCCs are specified. After migration along the dorsoventral pathway NCCs give rise to sensory and sympathetic neurons, Schwann cells, and endoneural fibroblasts among others. Dorsolateral migration leads to differentiation into melanocytes [19].

Founder melanoblasts, the unpigmented precursors of melanocytes, express microphthalmia-associated transcription factor (MITF), PAX3 and SOX10. Predetermination of NCCs into melanocytes in the MSA appears already by expression of KIT and MITF followed by dopachrome tautomerase (DCT) [20, 21]. Due to this early specification cells follow the dorsolateral migration path. Yet, also external factors could influence the migration of cells and the specification into the melanocytic lineage. Melanoblast migration is accompanied by high proliferation and inhibition of apoptosis at the same time. Signaling via the KIT receptor pathway is crucial in this phase. These unpigmented melanocyte precursors migrate a far distance along a rostrocaudal gradient and colonize the whole embryo. Then they enter the epidermis by crossing the basal membrane. Finally, these cells migrate into the hair follicle expressing tyrosinase (TYR) and tyrosinase related protein1 (TYRP1), enzymes involved in melanogenesis, leading to pigmentation. Some cells will give rise to melanocyte stem cells residing in the bulge region of the hair follicle while others differentiate and mature to fully functional melanocytes [22]. The fully differentiated melanocytes will produce melanin and transfer the pigment to keratinocytes which results in pigmentation.

V.3.1. Genes involved in melanocyte development

Several different molecules at various stages are necessary for differentiation of neural crest cells into melanocytes, as well as for proliferation and survival of the cells.

V.3.1.1. *WNT and BMP*

WNT (wingless integrated) and BMP (bone morphogenetic protein) molecules are present early in the dorsal neural tube. BMP leads to differentiation into neuronal and glial cells, while inhibiting development of pigment cells. WNT molecules are involved in the differentiation of two cell types arising from the neural crest. Signaling via the WNT pathway favors outgrowth of sensory neurons and melanocytes. NCCs which delaminate late from the neural crest give rise to melanoblasts. At this stage, BMP concentrations are low while WNT signaling is still present. WNT molecules and beta-catenin are crucial for induction of the neural crest and determination of melanocytic cell fate. WNT1 and WNT3 were found to be expressed in the dorsal part of the neural tube. During differentiation, these factors favor the development of pigment cells by directly activating MITF expression via beta-catenin [23].

V.3.1.2. *MITF*

MITF plays a central role in melanocyte development. It is a member of the basic-helix–loop–helix-leucine-zipper (bHLH-LZ) transcription factor family, which is essential for differentiation into the melanocytic lineage [24]. The bHLH-LZ structure is necessary for DNA binding and dimer formation. MITF binds as a homo or heterodimer to the Ebox motif CANNTG [25]. The gene consists of nine highly conserved exons. Alternative splicing leads to at least eight human isoforms of MITF with varying N termini. Each of the isoforms has a separate first exon which is controlled by a unique promoter region [26]. MITF-M is the melanocyte specific isoform which can directly regulate the main enzymes involved in melanogenesis: TYR, TYRP1 and DCT. Additional downstream targets of MITF are several melanocyte markers such as PMEL17, MELAN-A, MC1R, and AIM1. MITF itself is regulated by a variety of factors including SOX10, PAX3, beta-catenin and TCF/LEF as well as cAMP/CREB [25, 27]. Mutations in MITF lead amongst others to defective melanocytes and are associated with the auditory-pigmentation disorder Waardenburg syndrome 2 [28].

V.3.1.3. *PAX3*

The transcription factor PAX3 is essential for melanoblast survival and differentiation and thus for the expansion of the melanocyte precursor population. PAX3 belongs to the PAX (paired box) gene family whose members generally possess a paired box domain and a paired-type homeodomain. These conserved motifs are necessary for DNA binding. PAX transcription factors in general play important roles in embryonic development [29]. In the early NC, PAX3 is specifically expressed orchestrating the activity of other factors involved in NC differentiation [30]. Especially in the development of melanocytes PAX3 is a key player

during embryogenesis. It is involved in several stages such as specification, survival, proliferation and migration. Differentiation of cells into the melanocytic lineage is promoted by PAX3 due to transactivation of TYRP1. PAX3 alone can activate the MITF promoter, and together with SOX10 a synergistic activation can be achieved [31]. In humans, ten alternative splice transcripts of PAX3 exist. Out of these ten isoforms eight encode for proteins [32] which display different functions in melanocytes. PAX3 isoforms were shown to differentially regulate downstream targets which are involved in cell proliferation, migration, adhesion, apoptosis, angiogenesis and survival [29, 33]. For example the isoforms PAX3c, PAX3d and PAX3h were demonstrated to stimulate melanocyte proliferation. Negative regulation of cell proliferation in melanocytes was noted for PAX3a, PAX3b and PAX3e. No significant difference in cell proliferation was observed for PAX3g [33]. Transcriptional targets of PAX3 include MITF and TYRP1 [34]. Mutations in PAX3 can lead to various diseases including Waardenburg syndrome, craniofacial-deafness-hand syndrome, and alveolar rhabdomyosarcoma [35].

V.3.1.4. *SOX9 and SOX10*

Two proteins of the SOX (sex determining region Y-related HMG box) family of transcription factors, SOX9 and SOX10, have important functions in the development of melanocytes [36, 37]. They belong to the superfamily of high mobility group (HMG) box proteins that promote DNA binding through the HMG box domain in a sequence-independent, but conformation dependent manner. SOX9 and SOX10 belong to the SOX-E group, which additionally includes SOX8. SOX-E proteins promote the differentiation of NCC into various lineages [38, 39].

SOX9 plays a crucial role in the development of several tissues and cell types including various cells arising from the NC. Specifically in melanocyte development SOX9 stimulates the differentiation of melanocyte precursors [38]. Ectopic expression of SOX9 in NCCs favors the melanocytic cell fate. After differentiation of melanoblasts into mature melanocytes SOX9 expression is increased. Additionally expression of SOX9 is found in human melanocyte cultures and in melanoma cell lines with a pigment phenotype [37]. Further, the function of SOX9 is found to be controlled by signaling via the cAMP pathway. It is known that cAMP is important for the development and differentiation of melanocytes [40]. SOX9 might be activated by molecules which can increase cAMP concentration and in this way is involved in the induction of melanocyte cell fate and maturation.

SOX10 is suggested to influence the NC in different ways, such as facilitation of NC stem cell maintenance, but also specification and differentiation of distinct cell types [41, 42]. The expression pattern of SOX10 during development is highly dynamic, beginning in NC stem cells, being maintained during migration while it is lost upon differentiation in some cell types [37, 43]. SOX10 is known to be an important regulator of melanocyte differentiation by

promoting the survival and maturation of melanoblasts [43, 44]. During early development SOX10 controls the expression of MITF in the NC and thereby influences the activity of several genes crucial for commitment to the pigment cell fate and melanogenesis [45]. Importance of SOX10 expression during development and maintenance is shown as dysregulation of SOX10 is associated with Waardenburg syndrome type 4 [46]. Moreover, SOX10 transactivates DCT to stimulate its full activation together with MITF [47].

V.3.1.5. *LEF1*

Signaling *via* the WNT pathway is transduced by the transcription factor Lymphoid-enhancing factor-1 (LEF1). LEF1 can act as transcriptional repressor or activator depending on its binding partners. By binding to Groucho transcription is suppressed while interaction with beta-catenin leads to dissociation of Groucho from the promoter and thereby to initiation of transcription [48]. A functional binding site for LEF1 is present in the promoter region of the human MITF-M gene. *In vitro*, binding of LEF1 was confirmed which suggests a role in regulating MITF-M expression. Actually, binding of WNT3a to its receptor induces recruitment of LEF1 and beta-catenin to the MITF-M promoter and thereby increases transcriptional activity of this promoter [27]. Also binding of LEF1 to MITF via the bHLH-LZ region stimulates transcription from the MITF-M promoter. Yet, again beta-catenin is necessary for the collaborative function of LEF1 and MITF-M [49, 50]. Furthermore, LEF1 and MITF together can transactivate the promoter of DCT as well [50].

V.3.1.6. *FOXD3*

The transcriptional repressor FOXD3 is specifically expressed in precursors of neurons and glia cells while being absent in cells of the melanocytic lineage. FOXD3 is known to repress melanogenesis *via* MITF. By repressing the expression of MITF, FOXD3 inhibits the development of melanoblasts and can prevent migration along the dorsolateral pathway [51, 52].

V.3.1.7. *SCF/KIT and EDN3/EDNRB*

The stem cell factor (SCF)/KIT and the endothelin3/endothelin receptor B (EDN3/EDNRB) pathway are the two most important pathways promoting differentiation of NCCs into the melanocytic lineage [53]. At several stages in development, KIT plays an important role influencing migration and survival. Expression of KIT, as the expression of MITF, starts early in premigratory cells and is still present in migrating melanocytes [54]. EDN3 is involved in several functions such as migration, proliferation, survival and differentiation of melanocytes. It is already required very early in development for melanoblast migration. Expression of TYR is stimulated by EDN3/EDNRB signaling [55].

V.3.2. Signaling network in melanocyte development

As already indicated the cooperation of transcription factors during development is crucial for predetermination and full differentiation. MITF, the central factor in melanocyte differentiation interacts with several other factors, regulates their expression and is also regulated by several factors itself [56]. MITF expression can be stimulated via activation of the WNT signaling pathway. After translocation of beta-catenin into the nucleus, beta-catenin and TCF/LEF bind to the MITF promoter. In addition, both factors promote transcription of DCT [27, 50]. Binding of SCF to its receptor c-KIT results in the activation of MITF *via* the mitogen activated protein kinase (MAPK) pathway [54]. Further, cAMP is known to promote MITF expression *via* CREB, which is dependent on SOX10. Levels of cAMP can be increased by interaction of alpha-melanocyte-stimulating hormone (α -MSH) and melanocortin1 receptor (MC1R). [57]. Additionally, SOX10 and PAX3 synergistically bind to the MITF promoter and activate its transcription [31, 56, 58]. Activation of MITF leads to transcription of the melanogenic enzymes TYR, TYRP1 and DCT. Yet, also inhibitory factors are present which repress MITF expression. For example FOXD3 prevents MITF transcription by binding to PAX3 and thereby inhibiting its binding to the MITF promoter [52]. Further, a cross-regulatory interaction of MITF and SOX2 was suggested [59]. SOX2 could repress MITF and if SOX2 was absent MITF was expressed. On the other hand, ectopic expression of MITF led to suppression of SOX2. While PAX3 has a repressive function on DCT expression beta-catenin can end this suppression by inhibiting PAX3 [30]. For full activation of DCT synergistic binding of MITF and SOX10 is necessary [47]. SOX10 is also involved in the activation of TYR [60]. A schematic overview of the signaling network is given in Figure 1.

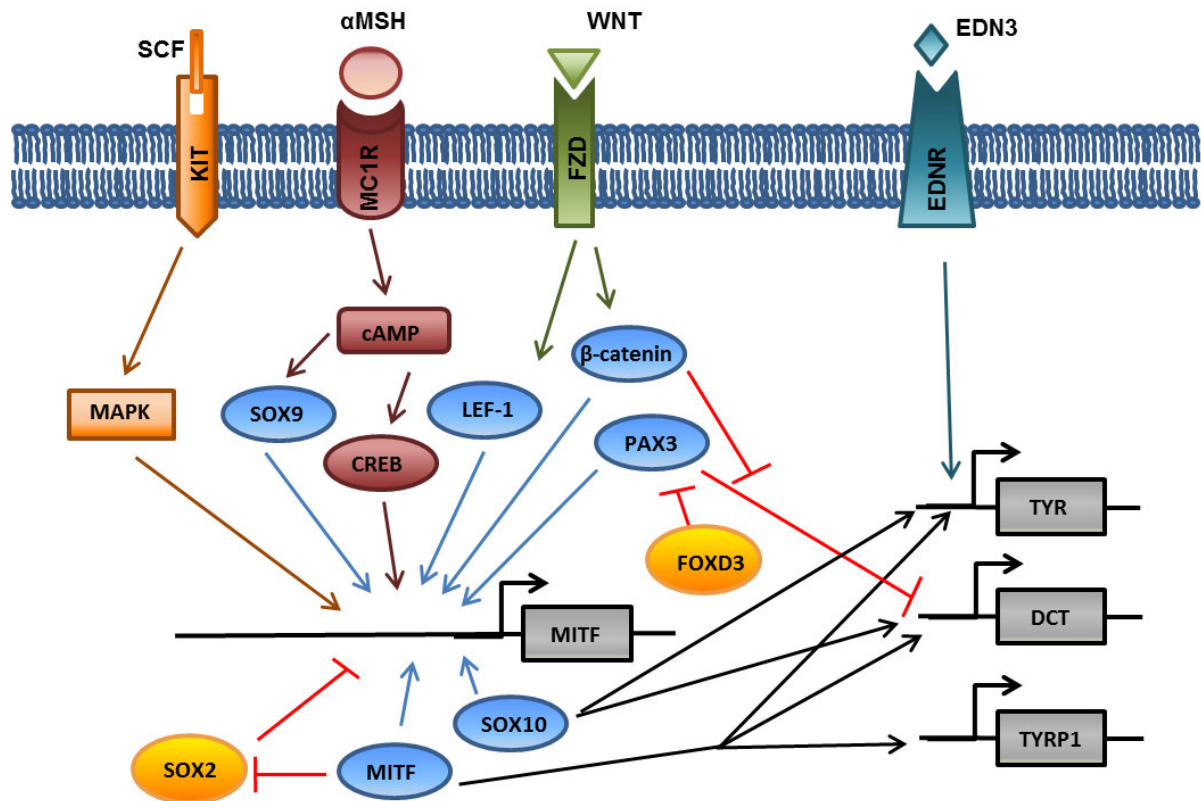


Figure 1: Signaling network in melanocytes for transcriptional activation of MITF and induction of melanogenesis. MITF is regulated by a variety of signaling pathways and factors. The main pathways include activation of MAPK by SCF/KIT, MC1R signaling via cAMP and CREB, WNT signaling via LEF1 and beta-catenin and EDN3/EDNR. Additional transcription factors, which are activating expression of MITF, are PAX3, SOX9, SOX10 and MITF itself. Negative regulation of MITF is induced by SOX2 and FOXD3. SOX2 directly suppresses MITF while FOXD3 functions via inhibition of PAX3 binding to the MITF promoter. MITF functions as key transcription factor inducing the three melanogenic enzymes TYR, DCT and TYRP1. Besides MITF, SOX10 can activate DCT and TYR. PAX3 represses DCT expression. Yet, this repression can be abolished by beta-catenin.

V.4. Melanocyte characteristics

Depending on the location, melanocytic cells possess unique functions and characteristics. In the inner ear melanocytes are essential for normal hearing by generating action potentials and play an important role in balance [61, 62]. Melanocytes present in the heart are suggested to have anti-inflammatory functions due to their reactive oxygen species (ROS) reducing activity [63]. The production of melanin in the brain, so called neuromelanin, protects the neurons by controlling ROS levels and reducing toxic compounds [64]. Besides that, melanocytes in the brain have neuroendocrine functions being involved in the regulation

of sleep and respiration [65]. However, the main and best-known function of melanocytes is the production of melanin, which is stored in specialized organelles called melanosomes [66].

V.4.1. Melanogenic enzymes

Several factors are involved in the synthesis of melanin, also known as melanogenesis whereby the three melanogenic enzymes, tyrosinase (TYR), dopachrome tautomerase (DCT) and tyrosinase-related protein 1 (TYRP1), play crucial roles [67]. Despite the high homology, each of them harbors its distinctive functional enzymatic activity. The enzymes regulate specific steps in the production of melanin, catalyzing different reactions [68, 69].

V.4.1.1. Tyrosinase

The rate-limiting enzyme in melanogenesis is TYR, a transmembrane copper glycoprotein which is essential for the initial steps of this process [70, 71]. The general structure and the active sites of this enzyme are highly conserved and it is expressed ubiquitously throughout the phylogenetic tree. First, it hydrolyzes tyrosine to L-3,4-dihydroxyphenylalanine (DOPA) and further oxidizes DOPA to DOPAquinone, which by itself oxidizes to DOPAchrome (Figure 2). The concentration and activity of tyrosinase is dependent on several factors such as UV radiation and stabilization by DCT and TYRP1 [70, 71]. In addition, the activity of tyrosinase influences the type of melanin produced. High tyrosinase activity results in eumelanin production while under low tyrosinase activity pheomelanin is generated. Mutations in the TYR gene result in Oculocutaneous albinism type 1 (OCA1) [72], a disease which is characterized by hypopigmentation of hair and skin as well as distinct ocular changes.

V.4.1.2. Dopachrome tautomerase

DCT, a type I membrane protein, is a member of the tyrosinase-related protein family and displays high homology to tyrosinase. Yet, instead of two copper ions in the catalytic center DCT harbors a zinc atom [73]. In contrast to TYR and TYRP1, DCT is already detectable in melanoblasts and therefore represents a melanoblast marker [22]. It catalyzes the conversion of DOPAchrome to 5,6-dihydroxyindol-2-carboxylate (DHICA) [68] (Figure 2). Mutations in the DCT gene can lead to variations in hair color by affecting the melanin synthesis [74].

V.4.1.3. Tyrosinase-related protein 1

The membrane protein TYRP1 is the most abundant glycoprotein exclusively expressed in melanocytes. The function of this enzyme is not completely understood, but it is supposed to harbor enzymatic activity [75, 76]. Yet, also the stabilization of TYR and the modulation of its catalytic activity were identified as main functions of TYRP1 [77]. Additionally, TYRP1 could influence the appearance of melanosomes [78]. Some mutations in the TYRP1 gene lead to

Oculocutaneous albinism type 3 (OCA3) [67]. Due to most of the mutations, however, a nonfunctional short version of the protein is produced resulting in hypopigmentation of skin, hair, and eyes [79].

V.4.2. Melanogenesis and melanin types

During the process of melanogenesis the pigment melanin is produced. Two types of melanin exist in the skin. Eumelanin is the brown or black melanin while pheomelanin is the red or yellow type [80]. Eumelanin is the most abundant melanin. It is a heterogeneous composition of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) forming a complex polymer containing nitrogen. Pheomelanin is also a nitrogenous polymer but mainly consists of 1,4-benzothiazine units which contain sulfur [81] (Figure 2). Melanin is produced and stored in melanosomes, which are specialized lysosome-related organelles found in pigment cells. These cytoplasmic vesicles contain the melanogenic enzymes TYR, TYRP1 and DCT. Two types of melanosomes exist according to the types of melanin produced: eumelanosomes contain the brown or black eumelanin and pheomelanosomes contain the red or yellow pheomelanin [82, 83]. Depending on the type, the organelles have an elliptical or spherical appearance, respectively. Typical for eumelanosomes is the fish net-like appearance. Intraluminal fibrils, mostly consisting of the transmembrane glycoprotein PMEL, are formed in premelanosomes that also influence the shape of the melanosome. During maturation, melanin accumulates on these fibrillary structures. Pheomelanosomes do not possess such fibrils and therefore have various shapes but are mostly round with a vesiculo-globular lumen [82, 84]. The distribution and amount of melanosomes determine the pigmentation of the skin. In dark pigmented skin high concentrations of eumelanosomes are present while only a few pheomelanosomes can be found.

Melanosome development can be divided into four stages. In stage I the melanosomes resemble multivesicular endosomes with luminal vesicles within an amorphous matrix. In this stage, matrix organization is initiated. In stage II, the matrix of eumelanosomes is organized in structured proteinaceous fibrils but melanin synthesis is not activated, yet. In contrast, melanin formation has started in pheomelanosomes already at this stage. Melanin deposition on the fibrils takes place in stage III. The matrix of melanosomes is fully melanized in stage IV [83].

The amount of free melanosomes in the epidermis is much higher in dark pigmented skin as well. Moreover, melanosomes are bigger and mostly present in mature stages [85]. UV exposure stimulates the transport of melanosomes inside the melanocytes along actin and microtubule filaments to the tips of the dendrites. Subsequently melanosomes are transferred

to the surrounding keratinocytes for UV protection preventing DNA damage and reducing skin cancer risk [83].

Different factors influence the type of melanin. In addition to the abundance and the activity of the melanogenic enzymes, especially tyrosinase, the melanocortin 1 receptor (MC1R) is a main factor determining eu- or pheomelanogenesis. Signaling *via* the MC1R favors generation of eumelanin due to stimulation of tyrosinase. Under low tyrosinase activity pheomelanin synthesis is favored, since only low amounts of L-dopaquinone are generated which are almost entirely used up for pheomelanin synthesis due to higher concentration of thiol compounds and the resulting conjugation of these two molecules [86]. Also other factors such as sulfur compounds, cytoplasmic cAMP level, WNT signaling and MAPK signaling can influence melanogenesis [71]. While eumelanin is thought to have a UV protective function [87] pheomelanin favors the production of reactive oxygen species which promotes melanomagenesis, thus the development of melanoma [88, 89].

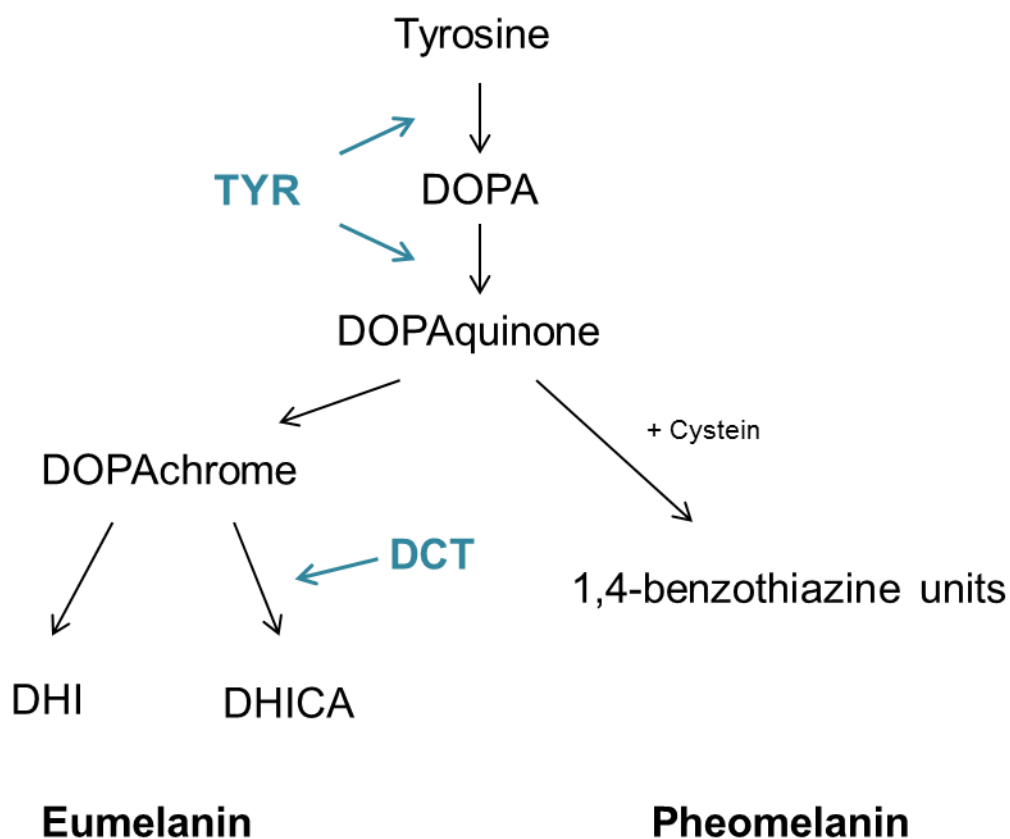


Figure 2: Simplified scheme showing parts of the melanin synthesis. The initial step of melanin synthesis is the conversion of tyrisone to DOPA and further to DOPAquinone. DOPAquinone can be converted into DOPACHrome leading to eumelanin production. Addition of cysteine to DOPAquinone leads to pheomelanin synthesis.

V.5. Skin Cancer

Skin cancer is the malignancy with the highest incidence in the US and Germany as well as many other countries with weakly pigmented populations [90]. Skin cancer can be divided into two main subtypes: Melanoma and non-melanoma skin cancer.

V.5.1. Non-melanoma skin cancer

The most common types of non-melanoma skin cancers include basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) which originate from dysregulated keratinocytes. About 75% of non-melanoma skin cancers are BCCs mostly arising at sun exposed areas [91]. Basal cells, non-keratinizing cells located in the deepest layer of the epidermis, undergo malignant transformation in this cancer type. Although these cells rarely spread to form metastases appropriate treatment is essential to prevent severe morbidity [92]. SCCs arise from keratinocytes residing in the epidermis and account for about 20% of the diagnosed non-melanoma skin cancers [93]. The development of SCC can also be correlated to sun exposure. As BCCs, SCCs usually do not form metastases. However, if they spread they migrate deep into the skin and can also gain access to the lymph nodes and proximal organs to form secondary tumors [92]. Rare types of non-melanoma skin cancers are Merkel cell carcinoma, cutaneous lymphoma, and Kaposi sarcoma which all together make up 1% of all non-melanoma skin cancers [93, 94].

V.5.2. Melanoma

Melanoma arises from melanocytes and can develop from abnormal moles. It is an aggressive skin cancer with high mortality rates for patients in advanced stages. Only about 2% of all skin cancers are melanomas yet it accounts for the majority of skin cancer related deaths in the US. The five year survival rate today lies at 98% for local malignancy in contrast to 63% and 16% if the cancer has metastasized to regional or distant areas, respectively [95]. Melanoma can be categorized into different subtypes. The three most common types, superficial spreading melanoma, nodular melanoma, and lentigo maligna melanoma, together account for 90% of melanomas diagnosed. The other 10% are acral lentiginous melanoma and other rare types [96]. The prognosis of the patient depends on the depth of invasion. Different staging systems can be used to estimate the stage of the patient. Today the Breslow scale and the mitotic rate of the primary tumor are considered most commonly. The thickness of the melanoma is measured by the Breslow scale [97]. The mitotic rate, which is determined histologically by the amount of mitosis per mm², is a measure for overall survival. Increased mitotic rates correspond to declined survival [98].

V.5.2.1. Genes involved in melanoma and SCC pathogenesis

Cancer development and progression can mostly be correlated with specific signaling pathways or distinct genes that are differentially regulated. Tumor promoting genes, also known as oncogenes, are often highly activated while tumor suppressor genes which can prevent tumor formation are downregulated or lost in cancer cells [99]. These changes in expression can be due to chromosomal aberrations, mutations or epigenetic modulations [100]. Certain pathways and genes have also been associated with the formation of SCC and melanoma.

V.5.2.2. Differentially regulated genes in SCC

In squamous cell carcinoma high rates of mutations in the tumor suppressor gene TP53 have been described. As the 'guardian of the genome' p53 is activated upon DNA damage, controls cell cycle progression and induces apoptosis. It is the most frequently mutated gene in SCCs with an incidence of 54%-69%. Already more than 50% of premalignant lesions carry this mutation [101, 102]. UV radiation causes the typical C-T transition mutation, which was detected at the TP53 locus in many actinic keratosis (AK) lesions, and SCCs. Mutations were mostly found in exon 5, 6, 7 and 8. Specifically codons 177, 179 and 241 were the hot spots in aggressive SCCs. [101, 103]. Thus, mutations in TP53 probably occur early in SCC development and UV exposure seems to play a crucial role in this process.

Additionally oncogenes are involved in the initiation and progression of SCC. For example, various mutations in the RAS gene have been described for SCC. These mutations lead to constitutive activation of the downstream signaling pathway resulting in uncontrolled proliferation due to cell cycle progression and resistance to apoptosis [104]. HRAS is commonly mutated by a substitution of glycine with valine at codon 12 (G12V). Additional RAS family members KRAS and NRAS are mutated less frequently in SCCs [105]. Yet, in AK mutations of HRAS or KRAS were reported at a frequency of 16%. Presence of these mutations in SCC precursor lesions indicates that RAS might be involved in the initiation of SCC [106].

V.5.2.3. Differentially regulated genes in melanoma

Altered signaling pathways accompany malignant cell transformation of melanocytes leading to melanoma formation. Growth factors become activated leading to uncontrolled proliferation accompanied by anti-apoptotic signaling and tumor suppressor inactivation. Prominent pathways known to be involved in melanoma initiation and progression are the classical MAPK or phosphoinositide 3-kinase (PI3K) and protein kinase B (AKT) signaling pathway. Classical oncogenes in melanoma are BRAF and NRAS. BRAF was found to be mutated leading to activation in most melanomas. Over 66% of these mutations result in a substitution of valine with glutamic acid at position 600 (V600E) leading to the activation of

the MAPK pathway [107]. NRAS is able to activate both, the MAPK pathway and the PI3K-AKT pathway as well [108]. BRAF and NRAS mutations are commonly found in the premalignant lesions of melanoma, so called melanocytic nevi, already [109, 110]. The BRAF V600E mutation was found in 18 out of 22 melanocytic nevi investigated. Mutations at codon 61 of NRAS were observed in three melanocytic nevi [109].

In addition to activation of oncogenes also loss of tumor suppressors can promote melanomagenesis. High expression of CDKN2B leads to growth arrest in nevus melanocytes and keeps these cells in a premalignant state. The loss of CDKN2B promotes to progression from benign melanocytic nevus to melanoma [111].

V.6. Transdifferentiation

Transdifferentiation is the direct conversion of one differentiated cell into another. During this process the cells acquire the phenotype of another cell type while losing the characteristics of the original one. This phenomenon is a subclass of metaplasia, which also includes switching of stem cells from one tissue type into another [112, 113].

V.6.1. Cell fate conversion

During embryonic development pluripotent stem cells differentiate into several distinct cell types of various tissue. Thereby their differentiation potential is reduced and they become committed unipotent cells of a certain lineage. The differentiation process was considered to be a unidirectional path which cannot be reversed. Waddington's epigenetic landscape model illustrated the concept of cell differentiation and lineage commitment [114]. The undifferentiated pluripotent cell is located above the somatic cells that are differentiated and specified to a certain lineage. When cells start to differentiate they are like 'a marble rolling down a hill', ending up in a certain valley representing final cell fate. However, advances in the stem cell field through generation of pluripotent cells by cell nuclear transfer [115], cell fusion [116], and reprogramming via ectopic expression of transcription factors [117] led to a paradigm shift. Cell differentiation can be reverted using different methods and is thus not a unidirectional process. Additionally, dedifferentiation into a precursor cell and transdifferentiation, the direct cell fate conversion from one somatic cell into another, was achieved (Figure 2). Already decades ago it was shown that the transcription factor MyoD (myoblast determination) can convert fibroblasts into muscle-like cells [118]. Following this, several direct conversions of closely related cells from the same germ layer have been reported [119-122]. More recently, even fibroblasts from the mesoderm could be transdifferentiated into ectoderm-derived neurons. This conversion between different germ layers was achieved by a combination of only three transcription factors: Ascl1, Brn2 and

Myt1l [123]. In addition to reprogramming this was another proof that ectopic expression of specific transcription factors is sufficient to change the cell fate of a committed cell.

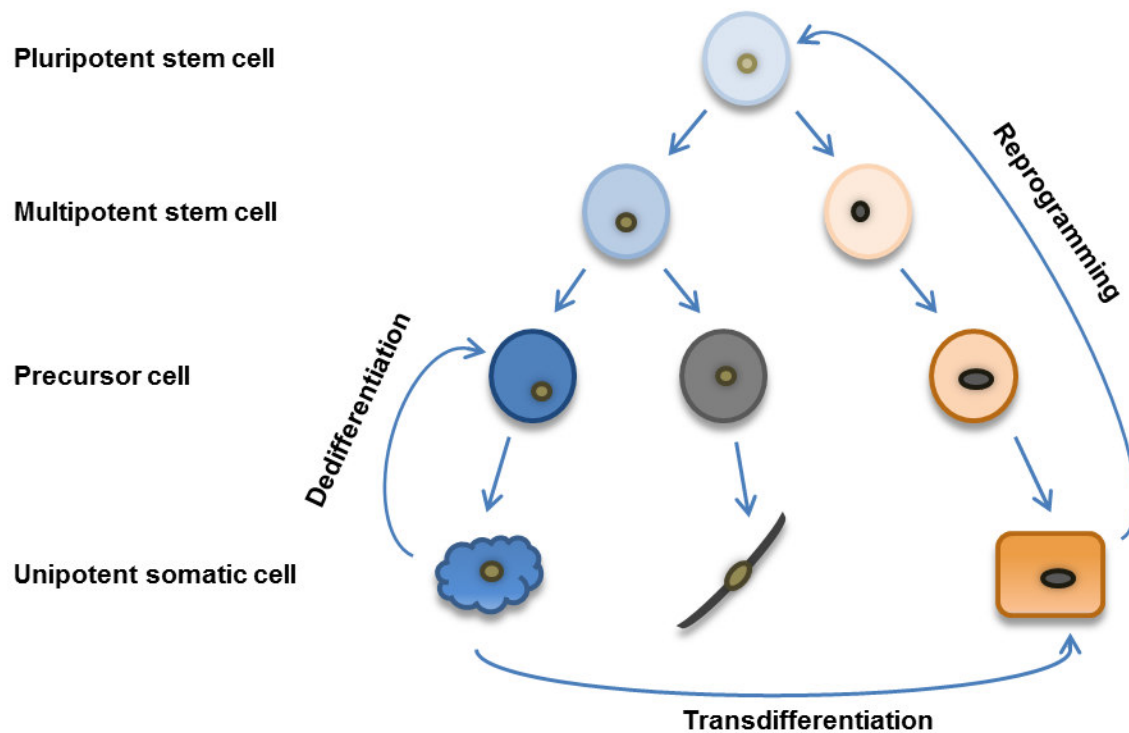


Figure 3: Cell fate conversion. Generation of pluripotent cells can be achieved by reprogramming of cells for example *via* ectopic overexpression of transcription factors. Dedifferentiation is the conversion into a precursor cell. Transdifferentiation is the direct switch from one differentiated cell type into another

V.6.2. Origin and definition of transdifferentiation

Transdifferentiation describes the (direct) conversion of one differentiated cell type into another [112, 113]. In nature, transdifferentiation occurs as a two-step process. The cells dedifferentiate first, start proliferating again and then differentiate into another cell type. One example is the regeneration of the newt lens [124]. Pigmented epithelial cells depigment and generate a dedifferentiated intermediate cell population, which can subsequently redifferentiate into primary lens fibers expressing crystalline. True transdifferentiation is defined by two criteria established by Eguchi and Okada. First of all, establishment of a lineage relationship between the two cell types is necessary. Secondly, cell types need to be easily distinguishable from each other. Changes in cell morphology alone are insufficient to proof transdifferentiation. Additionally, differences on the molecular level must be present [125, 126].

Experimental transdifferentiation, in contrast to natural transdifferentiation, is thought to be a direct process possibly with an unnatural intermediate state [127]. Often transcription factors known to play an important role in the development of the cell type of interest are used for

conversion. Ectopic expression of these factors activates the endogenous transcriptional program distinctive of the cell type, which should be generated. At the same time markers specific for the original cell type are downregulated. For example, CCAAT-enhancer-binding protein- α (CEBP α), a transcription factor inducing macrophage differentiation, can convert B cells into macrophages. During this transdifferentiation B cell markers such as PAX5 and CD19 are downregulated while MAC1, a gene specifically expressed in macrophages, is upregulated [119].

V.6.3. The role of epigenetic modifiers in cell fate conversion

Not only transcription factors but also epigenetic remodeling factors play an important role in cell type conversion. Changes in the epigenetic landscape during normal development stabilize cell fate and lineage determination [128-130]. Extreme changes in the epigenetic pattern also occur during reprogramming. Specific histone modification and demethylation can favor reprogramming. Treatment with the histone deacetylase inhibitor valproic acid (VPA) or 5-aza-cytidine (AZA), a DNA methyltransferase inhibitor promotes transition into a pluripotent state [131, 132]. The use of VPA allows reprogramming of human fibroblasts with only SOX2 and OCT 4. The two oncogenes c-MYC and KLF4 are not necessary for induction of the stem cell state [133]. Interplay between transcription factors known to be related to specific cell fates and epigenetic remodelers was suggested to be necessary for solid transition. Two specific methylation marks, histone 3 lysine 27 (H3K27) and histone 3 lysine 4 (H3K4) were identified to control reprogramming towards pluripotency [134, 135]. Epigenetic alterations were also detected during transdifferentiation. These changes are necessary for stable transdifferentiation in vivo and in vitro [136].

V.6.4. Purpose of studying transdifferentiation

Transdifferentiation is an interesting phenomenon to study for several reasons. First, investigating transdifferentiation will lead to a better understanding of the normal embryonic development since it will uncover key factors, including transcription factors, which are essential for the determination and differentiation of certain lineages. Moreover, identification of such key factors will help to understand the interplay between them providing insights into regulatory mechanisms and the hierarchical structure of transcription factor networks during development [137].

Secondly, advances in transdifferentiation could be used in regenerative medicine for cell replacement therapy. Specific cell types could be directly generated from patient cells. In several diseases cells are lost or damaged and cannot be replaced by the body itself. For example in type I and type II diabetes, the loss of functional β -cells leads to lack of insulin or insulin resistance, respectively [138, 139]. In 2008, Zhou and coworkers identified a transcription factor combination which transdifferentiated exocrine cells from the pancreas

into cells very similar to endocrine β -cells. These cells could produce and secrete insulin and thereby improve hyperglycemia in diabetic mice [120]. Other examples for diseases that could be treated by cell replacement therapy are neurodegenerative diseases like Parkinson's disease or Huntington's disease. For Parkinson's disease advances have been made in identifying the factors, which effectively generate dopaminergic neurons, by transdifferentiation [140].

Thirdly, transdifferentiation and metaplasia are closely related processes. Metaplasia is the abnormal transformation of one cell type into another, which is unusual for the type of tissue. Probably the best known example for metaplasia is the Barrett's metaplasia [141]. This transformation is suggested to predispose for cancer development. Therefore studying transdifferentiation can yield important information about tumor initiation and progression [137].

Finally, transdifferentiation has several advantages in comparison to reprogramming cells to pluripotency. Firstly, transdifferentiation is supposed to be faster and more efficient than reprogramming. To generate the cell type of interest via reprogramming the cells have to be dedifferentiated to create induced pluripotent stem cells (iPSCs). Thereupon, the cells then need to be redifferentiated. This procedure is time consuming and inefficient [142]. In 2013, Hanna and colleagues claimed to have increased the reprogramming efficiency to almost 100% by knocking out Mbd3, a core member of the Mbd3/NuRD (nucleosome remodelling and deacetylation) repressor complex. [143]. Yet, most of the established protocols used can convert cells much less efficiently. Transdifferentiation however, yields efficiency from up to 25% depending on the cell type [144, 145]. In addition, although iPSCs imply extensive clinical application, the potential is limited because of the possibility of tumor formation after transplantation. Due to their high differentiation potential, iPSCs can form teratomas, benign tumors of various tissue types, upon injection. These teratomas can gain malignant characteristics [146, 147]. Additionally, the expression of the oncogene c-MYC, which is used for reprogramming, could induce tumor formation [148]. Transdifferentiated cells do not harbor this risk because differentiated cells with limited differentiation potential are generated *via* this method. Further, only factors favoring the targeted differentiation are used which usually do not include oncogenes.

V.6.5. Progress in the field of transdifferentiation

Transdifferentiation has been intensively studied in the last few years. Many advances have been made and various cell lines could be generated by either intra-germ layer transdifferentiation from cells closely related but also transdifferentiation across germ layers was achieved. A recent comprehensive overview is given in the review by Xu and colleagues [149].

V.6.5.1. *Intra-germ layer transdifferentiation*

Within the mesoderm fibroblasts have been converted into cardiomyocytes and muscle cells. The factor combination GATA4, MEF2C and TBX5 was identified to generate cardiomyocyte-like cells [150]. For induction of cardiomyocytes *in vivo* the additional factor HAND2 was necessary [151]. Another example for transdifferentiation within the mesoderm is the cell fate switch from B cells and T cells into macrophages by either using the single factor CEBP α or CEBP β [119]. The transcription factor PU.1 was sufficient to achieve dendritic cells from T cells progenitors [152].

Induction of pancreatic β -cells *in vivo* is the most prominent example for transdifferentiation of cells within the endodermal lineage. In this example, the transcription factor combination NGN3, PDX1 and MAFA was expressed in exocrine cells from the pancreas. However, the efficacy of this combination seemed to be cell type specific since fibroblasts could not be converted using these factors [120]. Different neural subtypes have been generated by transdifferentiation more than 10 years ago. The expression of the transcription factors PAX6, ASCL1, NGN2 or DLX2, or combined expression in astrocytes led to the induction of neuronal cell fate [121, 153, 154].

V.6.5.2. *Transdifferentiation across germ layers*

After the break through study of Wernig and colleagues in 2010 several cell types have been generated across germ layers mostly by the conversion of murine or human fibroblast using a defined combination of transcription factors [123]. Besides converting fibroblasts into mature neuronal cells using ASCL1, BRN2 and MYT1L also neural stem cells were generated [155], representing transdifferentiation from cells of the mesoderm into ectodermal cell types. Transdifferentiation of mesodermal cells to endodermal cells has been achieved by the induction of hepatocytes from fibroblasts. Two different studies describe this process. The transcription factors GATA4, HNF1 α and FOXO3 combined with the knockout of p19Arf [156] or FOXA1, FOXA2, FOXA3 with HNF4 α were sufficient to induce hepatocyte cell fate [157].

Table 1: Overview of examples for transdifferentiation

Initial cell type	Target cell type	Transcription factors
Fibroblasts	Cardiomyocytes	GATA4, MEF2C, TBX5
B cells and T cells	Macrophages	CEBP α or CEBP β
T cells	Dendritic cells	PU.1
Pancreatic acinar cells	Pancreatic β cells	NGN3, PDX1, MAFA
Astrocytes	Neurons	PAX6, ASCL1, NGN2 or DLX2
Fibroblasts	Neurons	ASCL1, BRN2, MYT1L
Fibroblasts	Hepatocytes	GATA4, HNF1 α , FOXO3, knockout of p19Arf
Fibroblasts	Hepatocytes	FOXA1, FOXA2, FOXA3 with HNF4 α

V.6.6. Generation of melanocytes

Melanocytes can be found in several tissues of the body such as the eye, the inner ear, the hair bulge, the central nervous system and the skin. The main function of melanocytes is the production of pigment in the skin upon exposure to UV light and transfer to keratinocytes thereby preventing DNA damage in skin cells. Various pigmentary disorders have been described which are due to defects or loss of melanocytes [158]. Depigmentation of the skin due to loss of melanocytes (Vitiligo), for example, could be treated by transplantation of autologous melanocytes. Yet, clinical application is very low due to several drawbacks [159]. Thus, there is a need for an easily accessible source of patient-specific melanocytes.

V.6.6.1. Guided differentiation of stem cells into melanocytes

Generation of melanocytes was demonstrated for the first time by differentiation of murine embryonic stem cells (ESCs) in coculture with the stromal cell line ST2. The induction of melanocytes was dependent on the presence of steel factor and the efficiency could be enhanced by the addition of dexamethasone. At day 6 in culture, ESC colonies were visible. Between day 12 and day 13 some cells changed their morphology into spindle shaped dendritic cells and migrated out of the colonies. Expression of c-KIT and other melanocyte markers was detected. After 21 days the majority of the cells had turned into mature and fully pigmented melanocytes [160].

In 2006, Fang and coworkers achieved the differentiation of human ESCs into melanocytes. This differentiation process took 4-6 weeks. WNT3a, EDN3 and SCF were necessary for the induction of the melanocytic phenotype. Expression of the melanocyte markers MITF and TYR was activated and melanosomes filled with melanin were present in the cells. The transition was stable even after culture of these cells over a longer time and after transplantation into skin reconstruction. WNT3a was identified to be essential for induction of MITF and pigmentation of the cells. Reduction of WNT3a concentration caused a delay in

differentiation. The combination of WNT3a and EDN3 was sufficient for melanocyte cell fate induction. Additional factors which were added to the medium enhanced differentiation. A certain degree of differentiation could be achieved by growth factors alone. Yet, WNT plays an important role for the survival of NC progenitors. In conclusion, determination of the cell fate depends on the synergistic interplay of several factors [161].

Dermal stem cells (DSC) could be differentiated under the same conditions. Dendritic cells expressing melanocyte markers (MITF, DCT, S100, HMGB45) were generated. Culture of DSC in differentiation medium for two to three weeks was necessary to generate this phenotype. When transplanted into 3D skin reconstructs differentiated melanocytes settled on the basal membrane just like normal melanocytes. Melanocytic characteristics could also be induced by seeding DSC on skin reconstructs. Cells migrated into the epidermis and activated the melanocyte differentiation program [162].

IPSCs generated by three or four factors were differentiated into melanocytes by WNT3a, SCF, and EDN3. Using iPSCs circumvents the ethical issues connected to ESCs. Additionally, patient cells can be used for generation of the cell type of interest. First, embryoid bodies (EBs) were formed which were transferred to melanocyte differentiation medium containing WNT3, SCF, EDN3, cholera toxin (CT) after three weeks. Single cells were observed after one week under these culture conditions and pigmented cells were present after another 3-4 weeks. Melanocyte marker expression was activated and melanosomes were present as well. Additionally, these cells had lost their proliferative capacity which is also a typical characteristic for human melanocytes [163].

A cocktail of various cytokines was used in the previously described differentiation protocols to induce the melanocyte phenotype. Additionally, formation of EBs was necessary for dendritic cells to appear in culture. Nissan and colleagues described an elegant way to produce melanocytes from ESCs and iPSCs by only one factor. With a gradient of BMP-4 pigment cells (retinal pigment epithelium (RPE) and melanocytes) could be created from hESCs and iPSCs. Hereby it was possible to discretely investigate the different stages during development into mature cells. High concentration of BMP-4 induced the generation of epithelium and keratinocytes, while low concentrations were needed for neural cell differentiation. Using a BMP-4 gradient two subpopulations of pigment cells arose: RPE-like cells and bipolar cells expressing PAX3. The bipolar cells were further cultivated and after four passages melanocytes had developed. The gene expression profile resembled the one of melanocytes and the cells were positive for several melanocytic markers. Moreover, also melanosomes of all maturation stages were detected. Under coculture conditions with keratinocytes transfer of melanosomes could be observed. The generated melanocytes were

proven to be fully functional since they were able to cause pigmentation of cells in 3D skin reconstructs [164].

V.6.6.2. Transdifferentiation of fibroblasts into melanocytes

Tachibana and colleagues identified MITF as a critical factor in melanocyte differentiation. Morphological changes were induced in NIH3T3 mouse fibroblasts by ectopic expression of MITF. These cells adapted a dendritic morphology and expressed melanocyte markers and exhibited immature melanosomes in their cytoplasm. Yet, no synthesis of melanin could be noted, probably due to the mutated form of tyrosinase present in the parental cells. Therefore the generated cells resembled amelanotic melanocytes [165].

Recently, functional melanocytes have been generated from murine and human fibroblasts with a defined factor combination. After screening ten candidate factors, the three factor combination MITF, SOX10 and PAX3 has proved to be sufficient to activate the expression of several melanocyte markers and induce melanogenesis. In a 3D skin reconstruct as well as *in vivo*, the induced melanocytes were able to localize at the dermal-epidermal junction and transferred the produced melanin to neighboring keratinocytes. While murine fibroblasts changed their morphology after 14 days of induction and G418 selection, 40 days in culture were necessary for transdifferentiation of human cells with an efficiency of 40%. Marker expression was detected after 80 days. At day 100, 99.3% of the cells stained positive for TYR. The phenotype was stable and independent of transgene expression. Remarkably, conversion of adult human fibroblasts was much less efficient than conversion of fetal fibroblasts [166].

V.6.7. Transdifferentiation of cancer cells

In 2013, cancer cells have successfully been transdifferentiated by Thomas Graf's group. B lymphoma and leukemia cell lines were converted into macrophages *via* expression of the transcription factor C/EBP α . After induction cells downregulated B cell markers and expressed macrophage markers instead. Increased adherence, granularity and cell size was noted. Additionally, high phagocytic activity could be demonstrated. The induction also impaired the tumorigenic potential of these cells which was shown by injection into immunodeficient mice [167].

V.6.7.1. Correlation between tumorigenic potential and cell lineage

Recent studies suggest that the ability of cancer cells to form a tumor depends on the differentiation lineage. Reprogramming of cancer cells with subsequent differentiation into several cell types led to impairment of the tumor formation [168]. Also differentiation of so called cancer stem cells was efficient to repress the tumor phenotype [169]. Thus,

conversion of one cell type into another differentiation lineage could abrogate the tumorigenic potential of the cells. However, probably not only the conversion of cancer cells into another lineage underlies this phenomenon. Also terminal differentiation into postmitotic cells might have a crucial influence on the impairment of tumorigenicity. Generation of mature red blood cells and terminally differentiated connective tissue from sarcoma cells caused loss of tumorigenic potential of these cells. The suggested underlying mechanism is extensive epigenetic remodeling. It is known that differentiation is associated with epigenetic changes stabilizing the differentiation phenotype. Zhang and colleagues could show that reprogramming of cells into a dedifferentiated state is accompanied by significant epigenetic changes especially of tumor suppressor genes and oncogenes [168].

VI. Aims of the thesis

During development, lineage-specific transcription factors determine the cell fate. The ectopic overexpression of specific transcription factors was used for cell fate conversion. Several studies showed that distinct transcription factors can promote the transdifferentiation of one somatic cell type into another. Even the lineage of origin of cancer cells could be switched by this mechanism resulting in impaired tumorigenic potential. By directly converting cancer cells into other somatic cells, the mechanism by which normal cells turn into cancer cells can be better understood and the characteristics which are linked to the tumorigenic phenotype might be identified. This approach might additionally lead to an alternative therapeutical strategy for the treatment of cancer.

The aim of this project is to transdifferentiate tumor cells from the keratinocytic lineage into functional and potentially non-tumorigenic cells from the melanocytic lineage. We hypothesize that the tumorigenic potential of a cancer cell is dependent on the differentiation lineage. Thus, transdifferentiation of squamous cell carcinoma cells (SCCs) into the melanocytic lineage should yield non-tumorigenic cells.

The following research questions were addressed to test this hypothesis:

- 1.) Can SCCs be transdifferentiated into melanocyte-like cells by ectopic overexpression of a defined set of transcription factors?
- 2.) If so, have the resulting cells lost their tumorigenic potential?
- 3.) What is the mechanism behind the conversion into non-tumorigenic cells?

To answer these questions a transcription factor pool containing 21 factors was screened for the minimal combination of transcription factors necessary to convert SCC cells into melanocyte-like cells. Further, functional assays analyzing characteristics related to a malignant phenotype were performed with the transdifferentiated cells and their tumorigenic potential was investigated *in vivo*. Genetic alterations were investigated and the methylation landscape and the whole genome expression were analyzed *via* a gene set enrichment analysis to identify putative mechanisms responsible for the loss of tumorigenic potential upon transdifferentiation.

VII. Materials and Methods

VII.1. Materials

Table 2: Reagents and kits

Reagents and kits	Company
AlamarBlue	Invitrogen
Amersham ECL Prime Western Blotting Detection Reagent	GE Healthcare
Ammonium Persulfate Solution (APS)	Carl Roth
Ampicillin	Carl Roth
Apo-ONE® Homogeneous Caspase-3/7 Assay	Promega
Arcturus PicoPure RNA isolation Kit	Applied Biosystems
BrdU cell proliferation ELISA	Abcam
Calcium chloride	Carl Roth
cOmplete Mini Protease Inhibitor Cocktail	Roche Diagnostics
Cultrex 24 well BME Cell Invasion Assay	Trevigen
DH5α competent cells	Invitrogen
DNeasy Blood & Tissue Kit	Qiagen
dNTP	Bioron
Ethanol	Sigma-Aldrich
Human IL-24 ELISA	Cusabio
HumanCytoSNP-12	Illumina
HumanHT-12 v4 Expression BeadChip Kits	Illumina
ibidi Culture-Insert 500 µm	ibidi
Immobilion PVDF membrane pore size 0.45 µm	Merck Millipore
Infinium HumanMethylation450 BeadChip Kit	Illumina
Isopropanol	Sigma-Aldrich
LB (lysogeny broth) medium	Carl Roth
Methanol	Sigma-Aldrich
Phenol chloroform	Sigma-Aldrich
Phosphate buffered saline (PBS)	Biochrom
PhosphoStop Phosphatase Inhibitor Cocktail	Roche Diagnostics
Phusion polymerase	Thermo Scientific
Pierce BCA Protein Assay Kit	ThermoScientific
Plasmid Maxi Kit	Qiagen
Puromycin	Carl Roth
RevertAid First Strand cDNA Synthesis Kit	Thermo Scientific

RNase-free DNase Set	Qiagen
Rotiphorese Gel 30	Carl Roth
Skim Milk Powder	Sigma-Aldrich
Sodium acetate	Carl Roth
Sodium dodecyl sulfate (SDS)	G-Biosciences
SYBR Green PCR Master Mix	Applied Biosystems
Tetramethylethylenediamine	Carl Roth
Tween20	Applichem
X-tremeGene 9 DNA transfection reagent	Roche Diagnostics
Zeocin	InvivoGen

Table 3: Cell culture reagents

Cell culture reagents	Company
β-mercapthoethanol	Gibco® Life Technologies
Adenine	Sigma-Aldrich
Basic fibroblast growth factor (bFGF)	Promokine
Bovine pituitary extract (bPE)	Life Technologies
Braunol	Braun
Dimethylsulfoxide (DMSO)	Carl Roth
Dispase	Sigma-Aldrich
Dulbecco's Modified Eagle's medium (DMEM) AQmedia™	Sigma-Aldrich
Doxycycline	Sigma-Aldrich
Fetal Bovine Serum (FBS)	Biochrom
Forskolin	R&D Systems
Human melanocyte growth supplement (HMGS)	Gibco® Life Technologies
Hydrocortisone	Sigma-Aldrich
Insulin	Sigma-Aldrich
MCDB 153 media (MCDB)	Sigma-Aldrich
Medium 254	Gibco® Life Technologies
Penicillin/Streptomycin (Pen/Strep)	Sigma-Aldrich
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich
Recombinant human insulin-like growth factor-I	R&D Systems
12-O-tetradecanoylphorbol-13-acetate (TPA)	Sigma-Aldrich
Trypsin	Sigma-Aldrich

Table 4: Inhibitors

Inhibitors	Company
3-Methyladenine	Sigma-Aldrich
AKT Inhibitor VIII	Calbiochem
Mitomycin C	Carl Roth

Table 5: Primary and secondary antibodies

Primary and secondary antibodies	Company
Beta-actin	Cell Signaling
DCT	ProteinTech
IL-24	Abcam
MITF	Abcam
Pan-cytokeratin	Santa Cruz
Goat anti-mouse HRP	Cell Signaling
Goat anti-rabbit HRP	Cell Signaling

Table 6: Solution buffers and gels

Solution buffers and gels	
Phosphate buffered saline (PBS) pH 7.4	Tris buffered saline (TBS) pH 7.6
3.2mM Na ₂ HPO ₄	137mM NaCl
0.5mM KH ₂ PO ₄	20mM Tris
1.3mM KCl	
Running buffer pH 8.3	Transfer buffer pH 8.3
25mM Tris	25mM Tris
190mM glycine	190mM glycine
0.1% SDS	20% methanol
Laemmli 2x buffer pH 6.8	Washing buffer pH 7.6
4% SDS	137mM NaCl
10% 2-mercaptoethanol	20mM Tris
0.004% bromophenol blue	0.1% Tween20
0.125M Tris HCl	
Cell lysis buffer for protein isolation	
1x PhosphoStop	
1x cOmplete Mini Protease Inhibitor Cocktail	

1% Triton-X in TBS

Running Gel

3.3ml H₂O

3ml 30% acrylamide/bisacrylamide solution

2.5ml 1.5M Tris pH 8.8

100µl 10% SDS solution

100µl 10% ammonium persulfate solution (APS)

20µl tetramethylethylenediamine (TEMED)

Stacking Gel

3.4ml H₂O

0.83ml 30%acrylamide/bisacrylamide solution

0.63ml 1M Tris pH 6.8

50µl 10% SDS solution

50µl 10% ammonium persulfate solution (APS)

5µl tetramethylethylenediamine (TEMED)

Table 7: Analysis software

Analysis software	Source
7500 Software v2.0.5	Applied Bioscience
ApE	M. Wayne Davis (Open Source)
BD FACSDiva™	Biolegend
Chipster	Chipster Open source
Curve Expert 1.4	Cusabio
FlowJo 7.2.2.	FlowJo
Gene Set Enrichment Analysis (GSEA)	Broad Institute of MIT and Harvard
Genome Studio	Illumina
GraphPad Prism 5	GraphPad Prism
iControl 1.10	TECAN
ImageJ	National Institute of Health (NIH)
Ingenuity Pathway Analysis	Qiagen
Leica Application Suite v1.8.0	Leica
NIS-Elements Viewer	Nikon
RnBeads R-Package	Max Planck Institute Informatics Open source
T-scratch	CSElab

Table 8: Devices

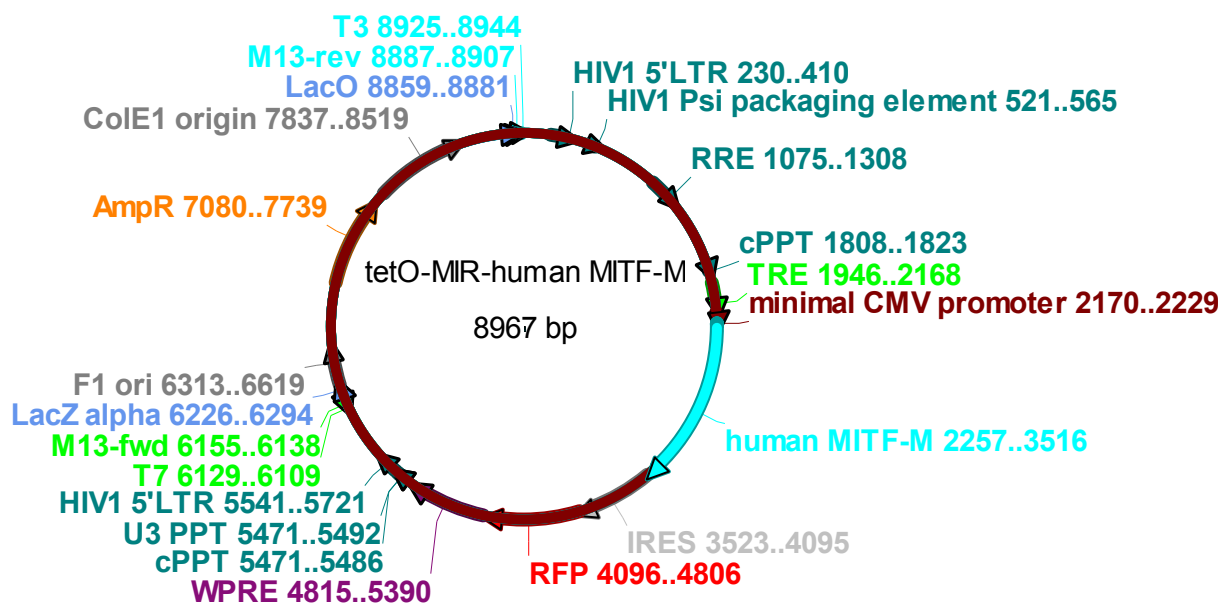
Devices	Company
AB 7500 Real Time PCR machine	Applied Biosciences
FACS LSR Fortessa	BD Biosciences
Leica DM LS light microscope	Leica
NanoDrop ND-1000 Spectrophotometer	Peqlab Biotechnologie GmbH
Nikon Eclipse Ti Fluorescence microscope	Nikon
Nikon Eclipse TS100 microscope	Nikon
TECAN infinite F200 pro microplate reader	TECAN

Table 9: Candidate transcription factors for transdifferentiation

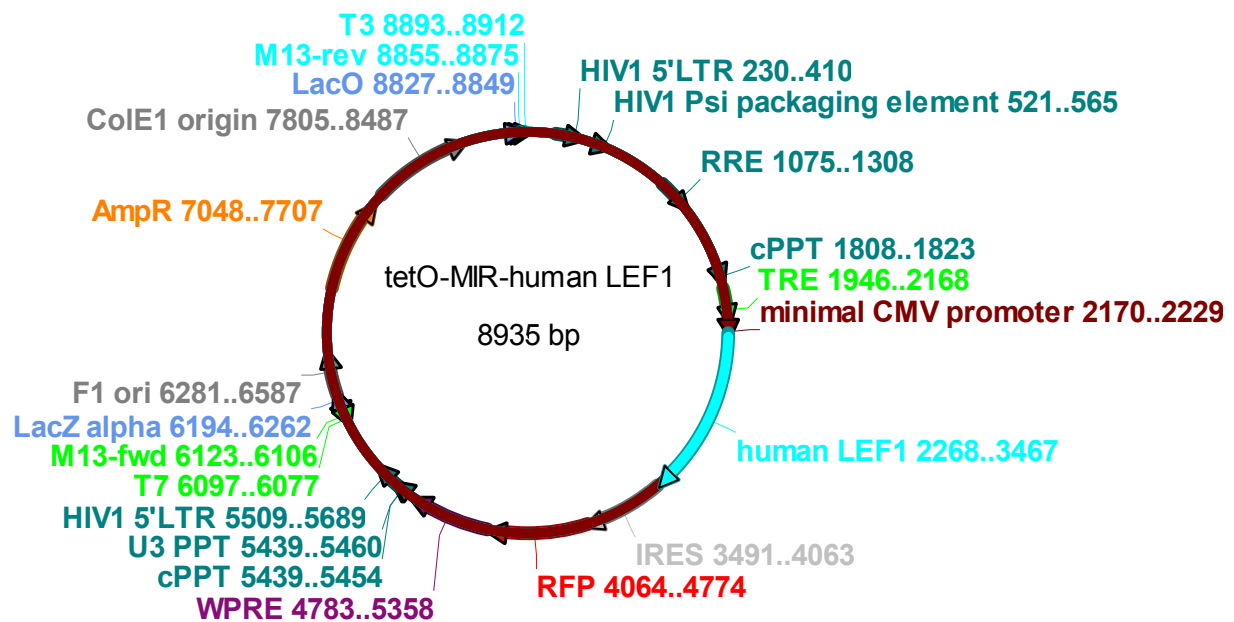
Candidate transcription factors for transdifferentiation	
MITF-M	NFIX
SOX2	IRF4
SOX5	ETS1
SOX9	FOSB
SOX10	HAND1
LEF1	HES1
Beta-catenin	HOXB7
PAX3	IFI16
SNAI2	KLF9
cMET	PITX1
TFAP2A	

VII.1.1. Plasmids

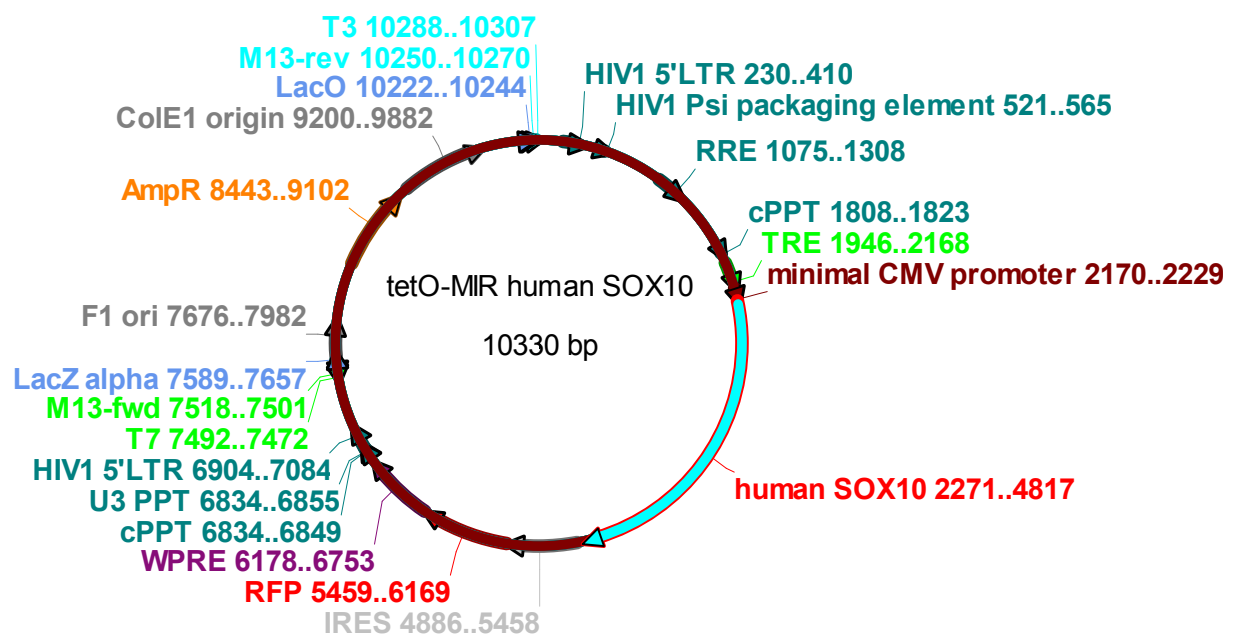
TetO-MIR human MITF-M



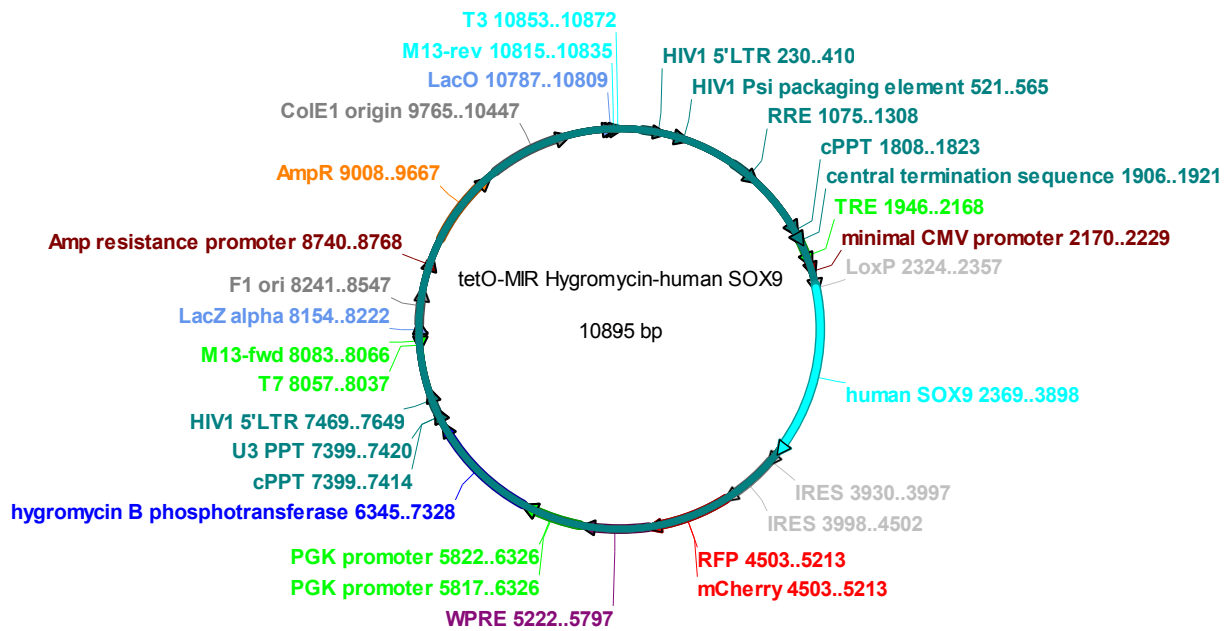
Tet-O MIR human LEF1



TetO-MIR human SOX10



TetO-MIR Hygromycin human SOX9



VII.2. 2.2 Methods

VII.2.1. Cell culture and cell lines

The SCC cell line MET-4 was cultured at 37°C in 5% CO₂ in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% Pen/Strep. This cell line was a kind gift from Prof. Petra Boukamp (DKFZ). HT-144 melanoma cells were cultured in DMEM with 10% FBS, 0.1mM β-mercapthoethanol, and 1% Pen/Strep. Transdifferentiated cells were cultured in MCDB 153 medium supplemented with 2% FBS, 20μg/ml bovine pituitary extract (bPE), 16nM 12-O-tetradecanoylphorbol 13-acetate (TPA), 1ng/ml basic fibroblast growth factor (bFGF), 5μg/ml insulin, 0.5μg/ml hydrocortisone, and 0.01mM forskolin.

VII.2.2. Isolation and cultivation of primary human melanocytes

Human melanocytes were isolated from foreskins, kindly provided by Dr. Uysal, Mannheim. Foreskin samples were incubated in 10% Braunol solution for 15min at room temperature. Subcutaneous fat was removed and the foreskins were cut into small pieces. During incubation at 4°C overnight in dispase (1 mg/ml) samples were digested. Digestion of the epidermis was performed with trypsin/EDTA for 15min at 37°C. Primary melanocytes were cultivated in medium 254 supplemented with 1% 100x human melanocyte growth supplement (HMGS) with a final concentration of 0.2% bovine pituitary extract, 0.5% FBS, 1μg/ml recombinant human insulin-like growth factor-I, 5μg/ml bovine transferrin, 3ng/ml

basic fibroblast growth factor (bFGF), 0.18 µg/ml hydrocortisone, 3µg/ml heparin, and 10ng/ml phorbol 12-myristate 13-acetate (PMA).

VII.2.3. Bacteria transformation

To amplify plasmid DNA DH5α competent *Escheria coli* (*E.coli*) bacteria were transformed. To this end, bacteria were thawed on ice and 10ng plasmid DNA was added. Bacteria and DNA was mixed gently before incubation on ice for 30min followed by heat shock at 42°C for 3min. After 2min on ice 500µl LB medium was added. Then the bacteria were shaken at 37°C for 30min. The transformed bacteria were spread on a selective agar plate and incubated overnight at 37°C for colony formation. Colonies were picked incubated in 5ml LB medium containing 100µg/ml ampicillin at 37°C overnight. The amplified plasmid DNA was isolated from this solution. Digestion with restriction enzymes was performed to confirm the presence of the specific plasmid. Bacteria containing the plasmid were incubated overnight on a shaker at 37°C in 200ml LB medium which contained 100µg/ml ampicillin. Subsequently the protocol of the Qiagen Plasmid purification Maxi Kit was followed to purify the plasmid DNA. After precipitation with isopropanol and ethanol plasmid DNA pellets were air dried and resolved in TE buffer. The NanoDrop ND-1000 was used to measure the concentration and for quality control.

VII.2.4. Lentiviral vector production and antibiotic selection

To produce lentiviral vectors, 770µl DMEM was mixed with 50µl Xtreme Gene9 and incubated at room temperature for 5min. Following the expression vectors encoding for the packing proteins *gag*, *pol* and *rev* and the envelop plasmid VSV-G in addition to the plasmid carrying the gene of interest were added. After an incubation of 30 min, the mixture was added to the HEK293T producer cells. After 12h, medium was changed. Medium was collected after 24h, 36h and 48h. The collected medium was filtered through a 0.45µm PVDF filter. Following, the supernatant was centrifuged for 5h 21,000rcf at 4°C in a high speed centrifuge and the virus was resuspended in PBS to store at -80°C. The concentrated virus was used to infect the cells. For the transdifferentiation assay, 4µl of centrifuged virus was added to the cells. The cells were washed with PBS before induction with doxycycline (1µg/ml). Cells were selected for M2 transactivator with 50µg/ml Zeocin and for the MITF reporter with 0.2µg/ml Puryomycin.

VII.2.5. RNA isolation, cDNA synthesis and quantitative RT-PCR analysis

RNA was isolated from the cells using the RNeasy Kit supplied by Qiagen following the manufacture's protocol. Briefly, cell pellets were lysed and RNA extracted using a column-based purification. DNA digestion was performed during DNase I treatment for 15min at room temperature on the purification column followed by two washing steps and the elution in RNase-free water. The RNA concentration and quality was measured at 260nm using a NanoDrop ND-1000 Spectrophotometer. The RevertAid First Strand cDNA Synthesis Kit was used for cDNA synthesis according to the manufacturer's advice, 500ng RNA was transcribed during 1h at 42°C followed by 5min at 70°C. The cDNA was diluted 1:5 in nuclease-free water and 2.5µl was used for analysis with quantitative RT-PCR (q-PCR) using SYBR Green PCR Master Mix and an Applied Biosystems 7500 Real-Time PCR System. The primer concentration used was 10mM. During q-PCR analysis, the expression of several keratinocyte markers and melanocyte markers was investigated. 18s was used as reference gene. Gene quantification was calculated using the Pfaffl method [170] calculating the delta-delta Ct. Statistical analysis was carried out in Excel.

Table 10: Q-PCR primers

	Forward primer	Reverse primer
MITF endo	ACCGTCTCTCACTGGATTGG	CGTTGGGCTTGCTGTATGTG
MITF total	TGCCTGTCTCGGGAACTTG	CCAGTGCTCTTGCTTCAGAC
SOX9 endo	AGACCTTTGGGCTGCCTTAT	TAGCCTCCCTCACTCCAAGA
SOX9 total	CTGAGCAGCGACGTCATCTC	GTTGGGCGGCAGGTA CTG
SOX10 endo	GGCTTTCTGTCTGGCTCACT	TAGAGGGTCATTCCTGGGGG
SOX10 total	AGCCCAGGTGAAGACAGAGA	ATAGGGTCCTGAGGGCTGAT
LEF1 endo	GCATCAGGTACAGGTCCAAGA	TCTCCAGAAGAGGTCTCTGGG
LEF1 total	AGAGCGAATGTCGTTGCTGA	TCTTGACCTGTACCTGATGC
MITF	GCTCACAGCGTGTATTTTTCC	GCTCACAGCGTGTATTTTTCC
DCT	GGTTCCTTTCTTCCCTCCAG	GGTTCCTTTCTTCCCTCCAG
TRP1	AGCAGTAGTTGGCGCTTTGT	AGCAGTAGTTGGCGCTTTGT
TYR	TTGTA CTGCCTGCTGTGGAG	TTGTA CTGCCTGCTGTGGAG
PAX3	CTGGAACATTTGCCAGACT	CTGGAACATTTGCCAGACT
EDNRB3	GTCCCAATATCTTGATCGCCAG	AAGGCACCAGCTTACACATCT
PMEL	TCTGGGCTGAGCATTGGG	AGACAGTCACTTCCATGGTGTGTG
c-KIT	TCATGGTCGGATCACAAGA	AGGGGCTGCTTCCTAAAGAG
FOXD3	TCTGCGAGTTCATCAGCAAC	GGGTCCAGGGTCCAGTAGTT
KRT10	GCTGACCTGGAGATGCAAAT	GCTGACCTGGAGATGCAAAT
KRT14	AGGAGGTCACATCTCTGGATGACTG	AGGAGGTCACATCTCTGGATGACTG
Integrin beta 4	CTGTACCCGTATTGCGACT	CTGTACCCGTATTGCGACT
Integrin alpha 6	GCTGGTTATAATCCTTCAATATCAATTGT	GCTGGTTATAATCCTTCAATATCAATTGT
Loricrin	GGAGTTGGAGGTGTTTTCCA	GGAGTTGGAGGTGTTTTCCA

Involucrin	CTCCATGTGTCATGGGATATG	CTCCATGTGTCATGGGATATG
IL-24	GACTTTAGCCAGCAGACCCTT	GGTTGCAGTTGTGACACGAT
SOX2	GCTAGTCTCCAAGCGACGAA	GCAAGAAGCCTCTCCTTGAA
18s	GAGGATGAGGTGGAACGTGT	GAGGATGAGGTGGAACGTGT

VII.2.6. Protein extraction and western blot

Cells were harvested in PBS and lysed in cell lysis buffer. Protein was harvested after centrifugation at 16,900rcf for 15min at 4°C. The concentration was measured using the Pierce BCA Protein Assay kit. Under non-reducing conditions 40µg protein were separated by SDS-PAGE on 12% gels. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes during wetblotting with 60V for 1h at RT. After blocking in 5% non-fat dried milk in TBST for 1h at room temperature, the membrane was incubated overnight at 4C° with the following primary antibodies diluted in 5% non-fat dried milk: MITF (abcam ab80651, mouse mAb 1:1000), DCT (protein tech, rabbit pAb 1:1000), IL-24 (abcam ab56811, mouse mAb 2µg/ml), beta-actin (Cell Signaling 13E5, rabbit mAb, 1:1000). Membranes were washed three times in TBS-T and subsequently incubated with a goat anti-rabbit or goat anti-mouse horseradish peroxidase linked antibody diluted 1:10000 in 5% non-fat dried milk in TBS-T at room temperature for 1h. Proteins were visualized by enhanced chemoluminescence (ECL) reagent as peroxidase substrate.

VII.2.7. Electron microscopy

Cells were seeded on aklar slides. Chemical fixation, dehydration, embedding, and imaging of the probes were performed by the Electron microscopy core facility at the DKFZ.

VII.2.8. Gene expression profiling by microarray analysis

Biological RNA triplicates of MET-4 cells and MT-MET-4 cells were sent to microarray analysis using Illumina HumanHT-12v4 Expression BeadChip according to the manufacturer's instructions, at the German Cancer Research Center (DKFZ) Genomics and Proteomics Core Facility. In the core facility quality control, reverse transcription with labeling, chip hybridization and calculation of mean averages was conducted for each probe. Raw microarray data was quantile normalized using Chipster. Probes with a fold change <2 and samples without annotation were removed before analysis. Following an empirical Bayes two groups test was conducted using to Benjamini-Hochberg (BH) p value adjustment with a threshold of $p < 0,05$. Hierarchical clustering was performed for the genes differentially expressed using Spearman correlation as a distance measure. Gene set test was performed

with Chipster using the Kyoto Encyclopedia of Genes and Genomes (KEGG) or Gene Ontology (GO) annotation gene lists with BH p value adjustment with a threshold of $p < 0.05$. The average linkage method was used to construct dendrograms out of the hierarchical clustering. Pathway analysis was conducted with Ingenuity Pathway Analysis (IPA).

VII.2.9. DNA isolation and Methylation array analysis

The Qiagen DNeasy Blood & Tissue Kit was used to isolate genomic DNA from MET-4 and MT-MET-4 cells according to the manufacturer's protocol. In short, genomic DNA was isolated from the mixture through column purification and eluted in buffer AE, after cell lyses and digestion with proteinase K for ten minutes at 56°C. DNA concentration measurement and quality control was performed using a NanoDrop ND-1000 spectrophotometer. DNA isolated from MET-4 cells and MT-MET-4 cells was sent to genome-wide methylation analysis using Illumina Infinium HumanMethylation450 BeadChips according to the manufacturer's instructions, at the German Cancer Research Center (DKFZ) Genomics and Proteomics Core Facility. Such an array contains 483,854 probes to investigate the methylation status of CpG sites located in CpG islands (CGIs), CGI shores, CGI shelves and open sea regions. Methylation of individual sites is calculated as beta-value ranging from 0% (not methylated) to 100% (fully methylated). Analysis of the methylation data and gene set enrichment analysis (GSEA) of the microarray data combined with the methylation array data was kindly performed by Olga Bogatyrova (Cancer Epigenomics, DKFZ) using RnBeads, an R package from the Max-Planck-Institute Informatik and GSEA software from the Broad Institute of MIT and Harvard, respectively. Inter- and intra-sample normalization was performed by raw data normalization *via* BMIQ-normalization using RnBeads R-Package (<http://rnbeads.mpi-inf.mpg.de/>). After quality control, the SNP probes (dbSNP132, $n=92,428$), probes with a p-value below 0.01 for at least one sample and probes with missing information were excluded. Also probes with methylation measurements in non CpG regions ($n=3,156$) were removed from the data set. Only weak batch effects were noted. Differentially methylated regions were analyzed on site and region level according to the sample groups specified in the analysis. The limma method was used to calculate the p value on the site level. The hierarchical linear model from R limma package were employed and fitted using an empirical Bayes approach on derived M-values. Differently methylated probes (DMPs) and regions (DMRs) were computed for MET-4 as increased or decreased compared to MT-MET-4 *via* the differently methylated module from RnBead R package, according to the package vignette (<http://rnbeads.mpi-inf.mpg.de/data/RnBeads.pdf>). Several metrics were used to compute the differential methylation on the site level. Based on the following three criteria each site was assigned a rank: 1) comparison of the differences in mean methylation

levels of the two groups 2) the quotient of mean methylation and 3) a statistical test (t-test or limma depending on the settings) estimating if the methylation values in the two groups arise from different distributions. A combined rank is computed as the maximum (i.e. worst) rank among the three ranks. Higher evidence for differential methylation is indicated by a small combined rank. Only sites and regions which have been tested significant were included in further analysis. GSEA was applied to test if the defined gene set of hyper- and hypomethylated genes in MET-4 vs. MT-MET-4 revealed statistically significant differences between the two biological groups and if they are in agreement with the whole genome expression. Analysis was done using GSEA v2.1.0 Release (<http://www.broadinstitute.org/gsea/>).

VII.2.10. Cell authentication and array CGH

Cell lines were authenticated using Multiplex Cell Authentication by Multiplexion (Heidelberg, Germany) as described recently [171]. Additionally, cell authentication was performed at the DKFZ genome core facility *via* comparative genomic hybridization (CGH) array analysis with the HumanCytoSNP-12 (SNP-Array with genome wide coverage) following the manufacturer's instructions. Analysis of the data was performed with GenomeStudio.

VII.2.11. Cell growth assay and BrdU cell proliferation ELISA

Cell growth was assessed by seeding cells in 6-well plates and counting them over a period of 16 days at 48h or 72h intervals. Additionally the proliferation of the cells was monitored by performing a BrdU assay following the manufacturer's protocol. After partial denaturation of the DNA strands immunochemical detection of BrdU is used to investigate the actively proliferating cells. Cells were seeded into a 96 well plate. After 24h BrdU was added and incubated for 24h at 37°C. Cells were fixed and DNA was denatured in one step by adding the fixing solution which was incubated for 30 min at room temperature. After washing the anti-BrdU detection antibody was added and incubated for 1h at room temperature. The peroxidase goat anti-mouse IgG conjugate was added and incubated for 30 min at room temperature. The TMB peroxidase substrate was pipetted on the plate and incubated for 30 minutes in the dark. After adding the stopping solution, the absorbance was measured immediately at 450/490nm using the TECAN infinite F200 pro microplate reader.

VII.2.12. Viability and drug sensitivity assay

To assess the cell viability, the alamarBlue cell viability assay reagent was used. This assay gives an indication for growth of the cells by detecting the metabolic activity. An oxidation-reduction agent is used for this purpose which fluoresces upon chemical reduction of the culture medium due to proliferation of the cells. Cells were seeded in 96 well plates in triplicates and incubated at 37°C. After 24h, 48h and 72h, alamarBlue reagent was added in a 1:10 dilution. For the drug sensitivity test cells were treated with 5mM or 10mM 3-MA (3-Methyladenine) or with 1µM or 10µM AKT VIII inhibitor. Some cells were also treated with a combination of both compounds. Cell viability was assessed after 48h of incubation. Cisplatin responsiveness was tested by treatment with 20µg/ml cisplatin for 48h. The fluorescence was measured with a TECAN infinite F200 pro microplate reader at 590nm 2h and/or 4h after addition of the solution. Cell viability was calculated from the resulting change in fluorescent intensity normalized to measurement at time point 24h 2h for cell viability, to the DMSO treated control in the drug sensitivity assay and to the untreated cells for cisplatin responsiveness.

VII.2.13. Apoptosis assay

The activity of caspase-3 and -7 was investigated using Apo-ONE® Homogeneous Caspase-3/7 Assay. These two effector caspases are activated upon apoptotic cell death and therefore can be used as indicators for apoptosis in mammalian cells. Briefly, cells were seeded in 96-well plates in triplicate. On the next day the caspase buffer substrate mixture was added. The buffer lysed and permeabilized the cells. The profluorescent substrate rhodamine110 becomes fluorescent upon sequential cleavage by caspas-3/-7. After 3h fluorescence signal was measured at 485nm excitation and 535nm emission using the TECAN infinite F200 pro microplate reader.

VII.2.14. Migration assay

To study the migration potential of the cells, ibidi culture inserts were used. The inserts were placed into 6-well plates and cells were seeded into the separated chambers to 90% confluency. After starvation overnight, the proliferation inhibitor mitomycin C (500µg/ml) was added (1:500 for MET-4 and 1:4000 for MT-MET-4), the inserts were removed and migration was observed after 5h, 10h and 24h for MET-4 cells and for MT-MET-4 cells. Quantitative data analysis was performed with the software TScratch.

VII.2.15. Invasion assay

The invasiveness of cells was analyzed using the Cultrex 24-Well BME Cell Invasion Assay according to the manufacturer's protocol. In short, transwell chambers were coated with 1x, 0.5x, and 0.1x BME (basal membrane extract) at 37°C overnight. Uncoated wells were used as migration control. After starvation with serum-free medium overnight, 100.000 cells per well were seeded in starvation medium in the top chamber. In the bottom chamber medium with 10% FCS was added as chemoattractant. The plate was incubated for 24h at 37°C. Migrated and invaded cells were lysed with a dissociation buffer containing Calcein, a fluorescent dye used for quantification. The TECAN infinite F200 pro microplate reader was used to measure fluorescence at 485nm excitation and 520 nm emission.

VII.2.16. IL-24 ELISA

To measure the secretion of IL-24, an ELISA assay was performed. The same amount of cells was seeded in 150µl in 96-well plates. After 5 days the supernatant was harvested and investigated. To the precoated ELISA plate sample or standard was added and incubated for 2h at 37°C. Without washing Biotin antibody was added. After 1h incubation at 37°C the plate was washed and incubated with HRP-avidin for 1h at 37°C. The substrate TMB was added. After 15min the reaction was stopped by the administration of the stop solution. The optical density was determined within 5min at 450nm with a correction at 570nm using the TECAN infinite F200 pro microplate reader. The concentration was calculated with the software Curve Expert 1.4.

VII.2.17. In vivo tumorigenicity assay

To investigate the tumorigenicity $1,5 \times 10^6$ cells were resuspended in 50% Matrigel and injected subcutaneously into the flank of NSG mice. This mouse was developed by JAX Professor Leonard D. Shultz and lacks mature T cells, B cells, and is deficient for several high-affinity receptors for cytokines (including IL2, IL4, IL7, IL9, IL15, and IL21) that block the development of NK cells and further impair innate immunity. Consequently, the NSG mouse engrafts human cells and tissues better than any other published mouse strain [172]. Per cell line four mice were injected. Tumor formation was observed over 30 weeks. Mice were sacrificed as soon as the tumor reached a size of 1cm in diameter or if the mouse showed behavioral abnormalities. Tumors were fixed in 4% Paraformaldehyde (PFA) overnight and embedded in paraffin. Samples were sliced, cleared with xylene and rehydrated before antigen retrieval. Staining was performed with Pan-Cytokeratin (1:300) and counterstained with haematoxylin. Additionally a haematoxylin & eosin staining was performed for

histological analysis. The Leica DM LS light microscope was used for analysis of the samples. Sayran Arif-Said embedded the fixed samples in paraffin and performed the cutting, deparaffinization and staining of the slices.

VII.2.18. Statistical analysis

The software GraphPad Prims version 5.00 was used for statistical analysis. Statistical significance using two-tailed paired and unpaired t-test, two-way ANOVA, Mann-Whitney U test or Fisher's exact test was determined if $p < 0.05$ (*), <0.01 (**) and 0.005 (**).

VIII. Results

VIII.1. Identification of transcription factors which promote transdifferentiation into the melanocytic lineage

In normal development and differentiation the specific interaction of several factors is necessary to determine cell fate. Therefore, we hypothesized that not only a single factor but a factor combination is necessary for transdifferentiation of cells from the keratinocytic into the melanocytic lineage. We had a pool of candidate genes which included transcription factors being involved in NC development, melanocyte differentiation, and which are highly expressed in melanocytes or upregulated during differentiation of iPSC into melanocytes. The candidate genes were cloned into doxycycline-inducible lentiviral vectors that co-expressed red fluorescent protein (RFP) in order to monitor transgene expression. A reporter construct which contained green fluorescent protein (GFP) under the control of the melanocyte-specific MITF promoter region was used to detect expression of endogenous MITF. This MITF reporter construct was selectable with puromycin (Figure 4A). Additionally, co-infection with a lentiviral construct expressing the reverse tetracycline-controlled transactivator (rtTA or M2) was necessary to induce transgene expression. Cells, which carried this vector, were selectable with zeocin. Before testing various transcription factor cocktails cells were transfected with the M2 rtTA and the MITF reporter construct followed by antibiotic selection with zeocin and puromycin. After two weeks of antibiotic treatment all cells in this culture contained these two constructs (Figure 4B).

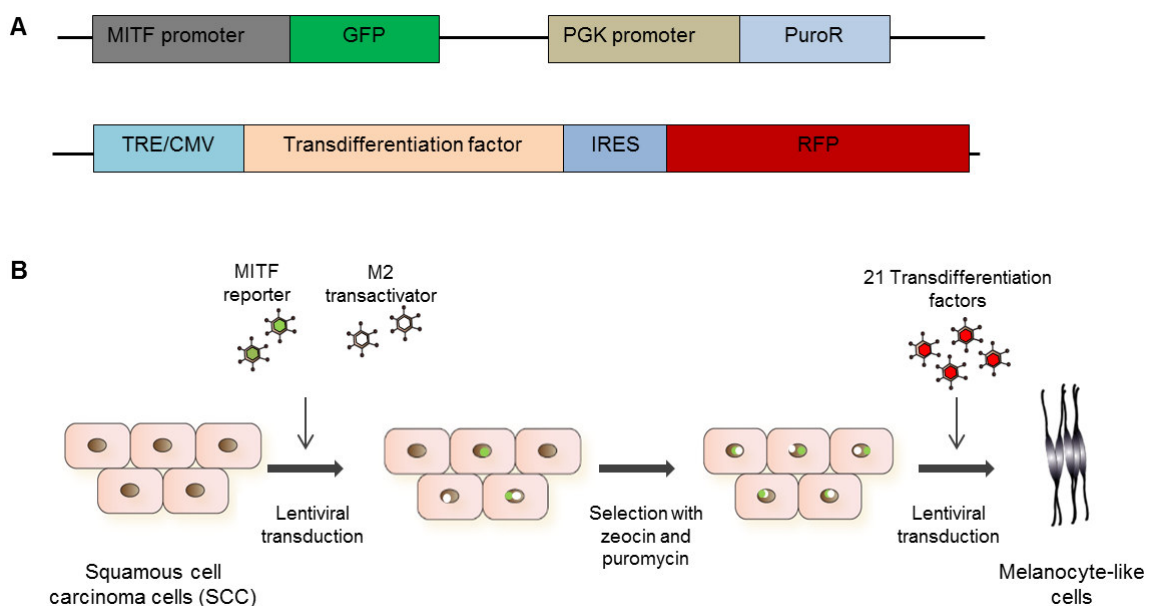


Figure 4: Schematic representation of the lentiviral vector constructs and workflow

A) Schematic representation of the lentiviral vectors carrying the MITF reporter construct and the transgenes used for transdifferentiation. The MITF reporter construct was used to monitor endogenous MITF expression. To this end, the MITF promoter region was coupled to a GFP site. Cells containing this vector could be selected with puromycin. The vectors containing the transdifferentiation factor carried a RFP site to make transgene expression visible under the fluorescence microscope. (GFP: green fluorescent protein, PGK: phosphoglycerate kinase, PuroR: puromycin resistance, TRE/CMV: tetracycline responsive element/cytomegalovir, IRES: internal ribosomal entry site, RFP: red fluorescent protein). B) Workflow. Squamous cell carcinoma cell line MET-4 was transfected with the M2 transactivator and the MITF reporter construct. Cells were selected with zeocin for the M2 transactivator and with puromycin for the MITF reporter construct. After two weeks of antibiotic selection, cells were transfected with the transdifferentiation factors.

For the validation of the MITF reporter construct primary human melanocytes and MET-4 cells were transfected with this vector and GFP expression was observed. Strong GFP expression was noted for melanocytes due to high MITF expression. No GFP signal was detected in MET-4 cells since they do not express MITF (Figure 5A). The expression of the single transgenes was demonstrated *via* q-PCR. Endogenous expression as well as total expression was analysed in cells transfected with single factors uninduced or after induction with doxycycline. No endogenous expression was present for MITF, SOX10 and LEF1. Induction of the transgene led to a strong increase in total expression for these three genes. MITF expression was increased approximately 17-fold, SOX10 expression was almost 125-fold enhanced, and LEF1 mRNA level was 122-fold higher due to transgene expression. SOX9 was already highly expressed in MET-4 cells so that the induction of the transgene did not significantly increase the overall expression (Figure 5B-E).

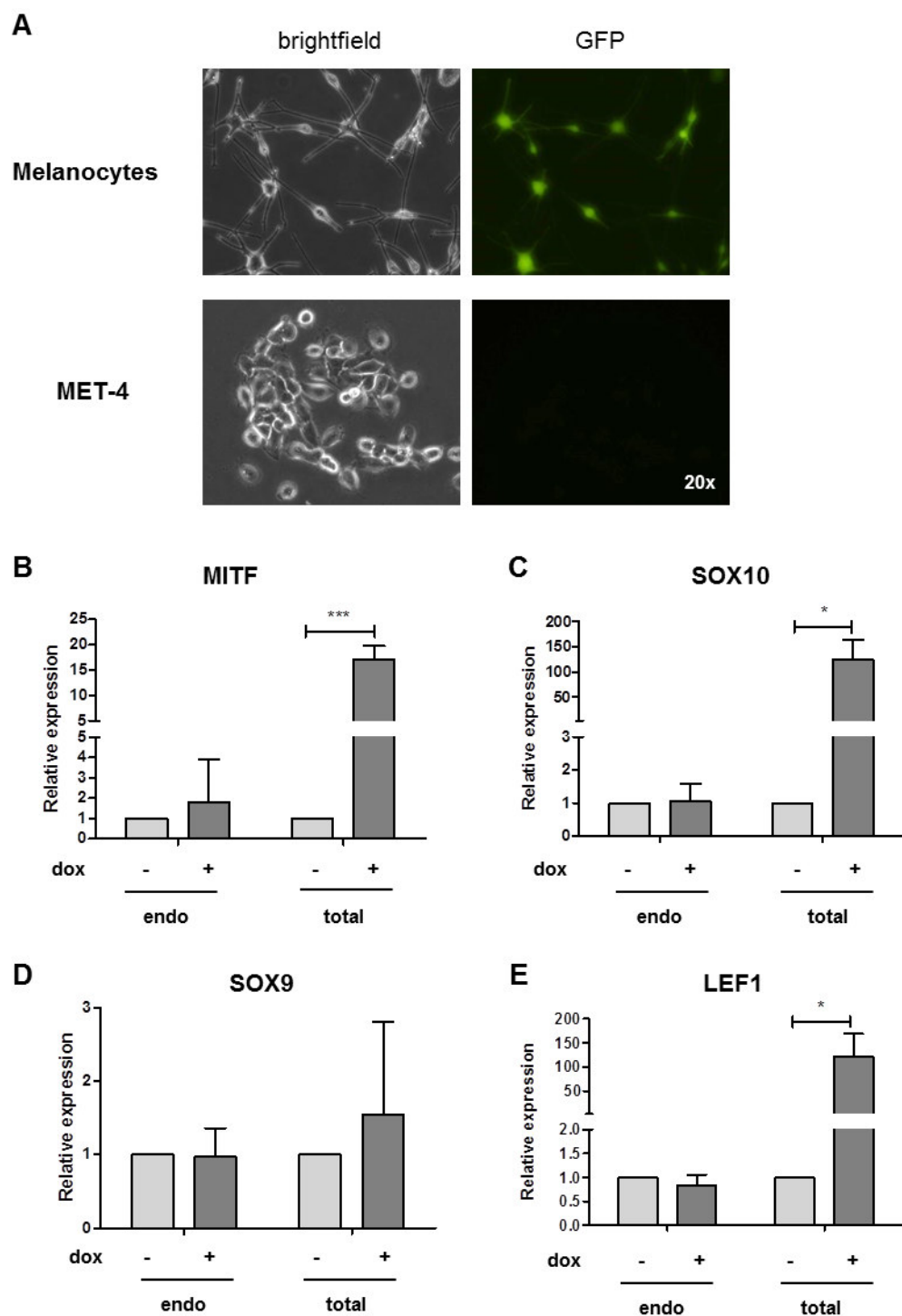


Figure 5: MITF reporter construct and transgene validation. A) Melanocytes and MET-4 cells were transfected with the MITF reporter. A strong GFP signal, which indicates MITF expression, was visible in melanocytes. In MET-4 cells MITF is not expressed and no GFP signal was detected. B-E) To validate the transgene vectors, MET-4 cells were transfected with single factors and expression was induced by doxycycline. The endogenous (endo) and total expression is calculated relative to the expression of the uninduced counterpart. Error bars indicate the SD of three independent experiments. P values were calculated by two-tailed, unpaired sample t-test. Asterisks indicate t-test p-value <0.05 (** p value < 0.01, *** p value < 0.005).

Cells were transfected with different combinations of transcription factors and the expression was induced by doxycycline. Two to three days after induction transgene expression could be observed by detecting RFP signal. Endogenous MITF expression indicated by GFP was visible approximately one week after induction (Figure 6A). In total cells were treated with doxycycline for two weeks before withdrawal. The medium was changed from DMEM to melanocyte differentiation medium MCDB one week after induction.

Transgene expression was observed for all combinations whereas the RFP signal intensity varied according to the amount of virus used (Figure 6B). Not all factor combinations could induce GFP expression representing no endogenous MITF in these cells. The combination of MITF, SOX9 and SOX10 induced only weak GFP expression. Addition of LEF1 seemed to increase MITF expression (Figure 6B). Thus, the combination MITF, LEF1, SOX9 and SOX10 (MLS9S10) resulted in the highest GFP expression.

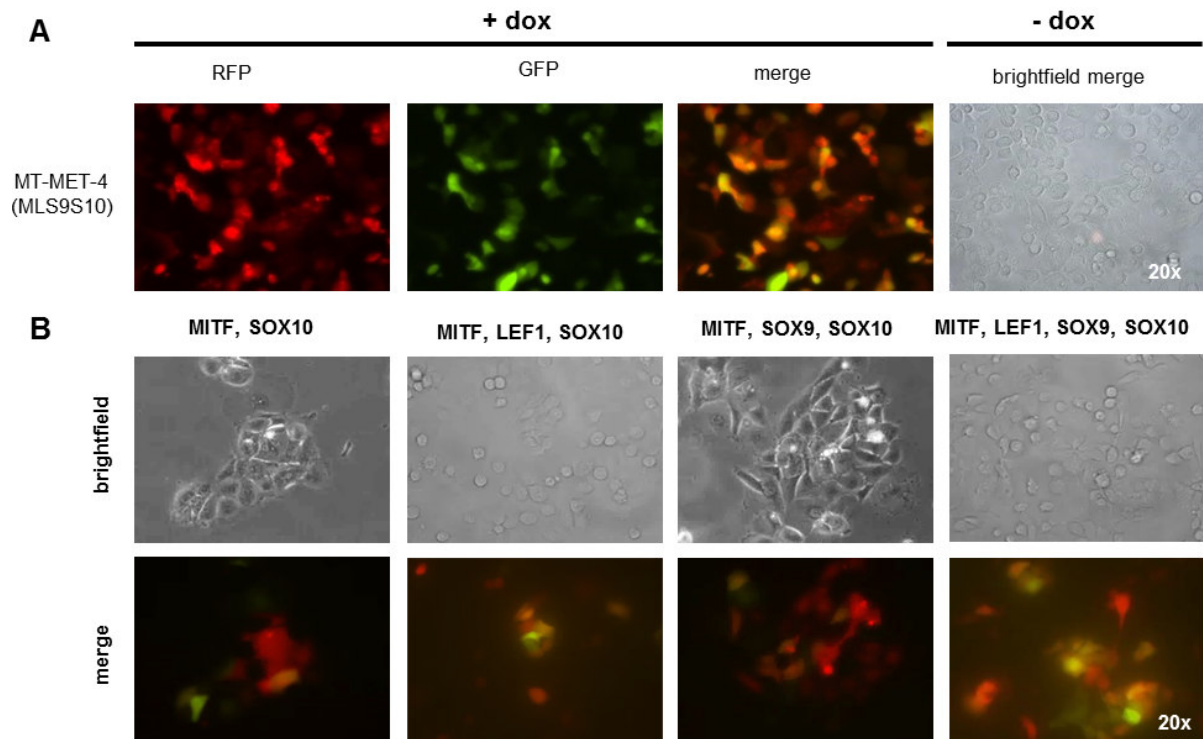


Figure 6: Transgene expression and reporter activation. A) The transdifferentiation cocktail activated a strong RFP signal in the cells. Also several of these cells were GFP positive. Neither a RFP nor a GFP signal was detected in uninduced cells. B) Other transcription factor combinations could induce MITF expression in MET-4 cells as well. Yet, the GFP signal seemed strongest with the final transdifferentiation cocktail MITF, LEF1, SOX9, SOX10 (MLS9S10).

Besides induction of endogenous MITF expression, several transcription factor combinations provoke morphological changes. Cells lost their cobblestone-like morphology and resembled melanocytes. Spindle shaped cells were present in culture which in addition lost the tight contact to the neighbouring cells. The amount of transdifferentiated cells varied depending

on the transcription factor combination used. Ectopic overexpression of solely MITF and SOX10 yielded only few spindle shaped cells. Addition of LEF-1 and SOX9 increased the transdifferentiation efficiency markedly. With the factor combination MLS9S10 approximately 80% of the cells changed their morphology (Figure 7A). However, the transdifferentiated cells were quickly overgrown by the cells that did not change their phenotype. Therefore, the isolation and separate enrichment of the transdifferentiated cells was necessary. MET-4 cells attach strongly to the bottom of cell culture flask. The transdifferentiated cells could be enriched by serial trypsinization since the melanocyte-like cells detached faster than the original MET-4 cells. Comparison of the morphology of melanocyte transdifferentiated MET-4 cells (MT-MET-4) to melanocytes showed obvious similarities (Figure 7B).

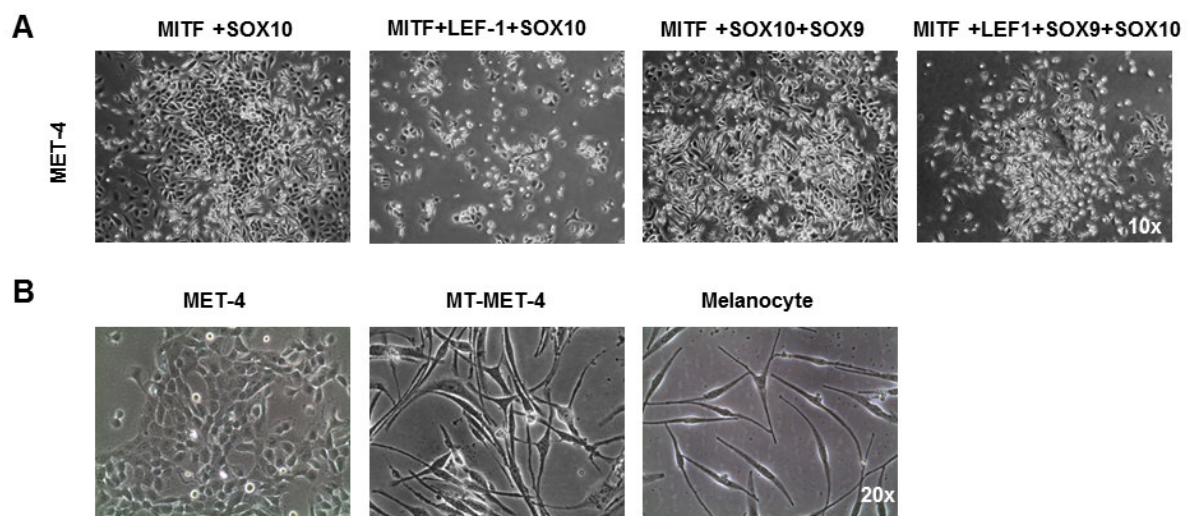


Figure 7: Morphological changes. A) The transcription factor combinations which were able to induce MITF expression additionally led to morphological changes in the cells. Cells adapted spindle shaped morphology and lost the tight cell contact to neighbouring cells. The combination of MITF and SOX10 could induce these changes already, however only in a low cell number. Highest amount of cells with changed morphology was present in the culture which was transfected with the MLS9S10 factor combination. B) Morphology of MET-4 cells, transdifferentiated MT-MET-4 cells and melanocytes. MET-4 cells showed cobblestone-like morphology and grew in tight colonies. The transdifferentiated MT-MET-4 cells adapted an elongated spindle shaped phenotype and lost the tight interaction with other cells comparable to melanocytes. Melanocytes were spindle shaped cells which grew as independent cells with only loose contact to other cells.

After withdrawal of doxycycline, transgene expression was undetectable whereas the morphological changes remained (Figure 8A). Thus, the phenotype seemed to be transgene-independent and therefore stable. MITF was described to be able to induce melanocytic characteristics in murine fibroblasts [165]. Transfection of cells with the transgene MITF alone was performed to test the effect of MITF overexpression in MET-4 cells. After induction of MITF transgene with doxycycline, several cells showed transgene expression indicated by

red fluorescence. However, ectopic expression of MITF alone was not sufficient to induce neither GFP expression nor morphological changes in MET-4 cells (Figure 8B, upper row). Thus, although MITF is the key player in melanocyte development and differentiation other factors were necessary for transdifferentiation. Since several cytokines which favour melanocyte differentiation are present in the MCDB medium MET-4 cells were cultured in this medium to test if the medium alone could induce melanocytic characteristics. However, MET-4 did not survive under these conditions (Figure 8B, lower row).

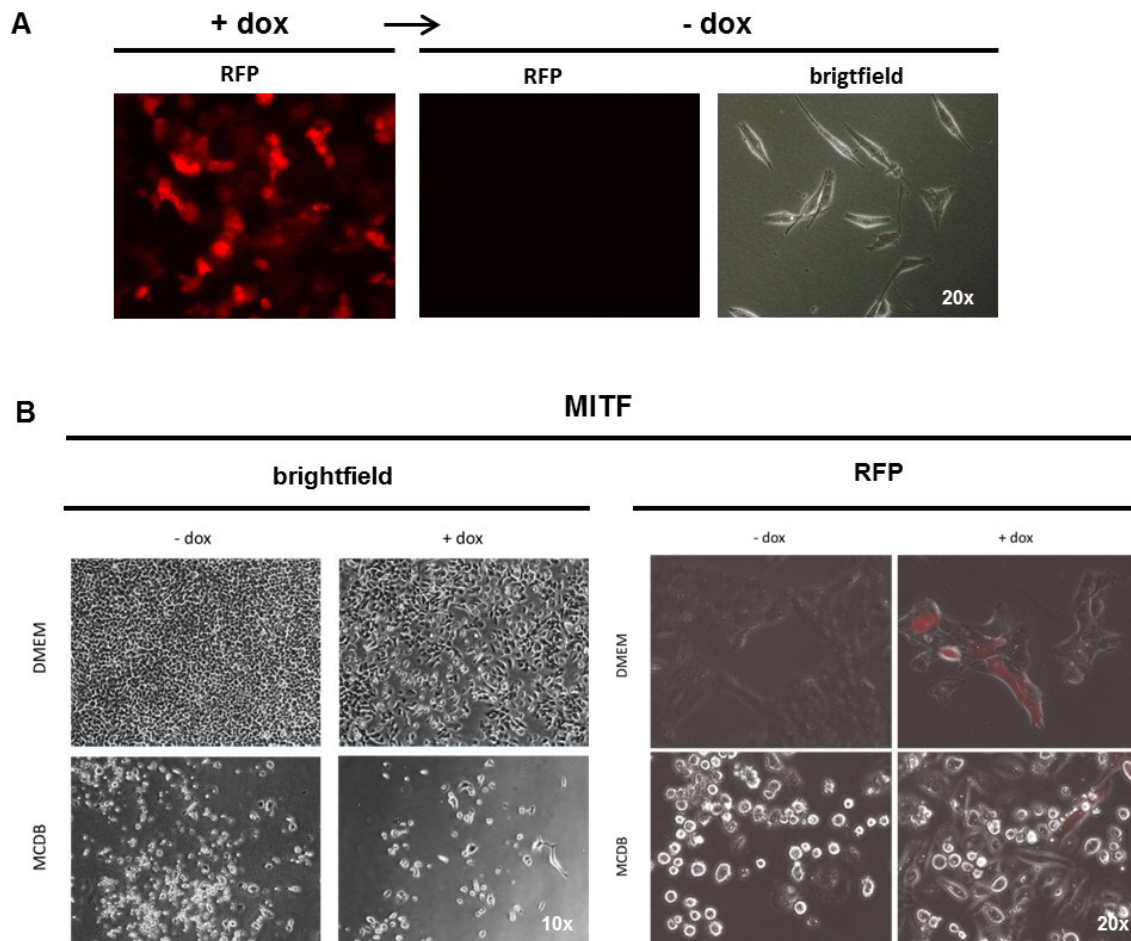


Figure 8: Effect of MITF and doxycycline withdrawal. A) After transfection with transgenes and induction with doxycycline strong transgene expression could be detected indicated by red fluorescence. After doxycycline withdrawal transgene expression was undetectable after one week but the cells kept their altered morphology. B) MET-4 cells were infected with MITF alone, cultured in DMEM or MCDB with and without induction with doxycycline. MET-4 cells cultured in DMEM grew normally. After induction transgene expression was observed however neither endogenous MITF expression nor morphological changes were noted. MET-4 cells did not survive culture in MCDB medium.

VIII.2. MT-MET-4 cells acquired melanocytic cell characteristics

After enrichment of the transdifferentiated cells by serial trypsinization marker expression was analysed *via* q-PCR. All keratinocyte markers tested including Keratin10 (KRT10) and Keratin14 (KRT14), Integrin beta4 and Integrin alpha6 as well as Loricrin and Involucrin, were significantly down regulated in the transdifferentiated MT-MET-4 cells compared to parental MET-4 cells (Figure 9A). The expression in MT-MET-4 cells was reduced by two thirds for KRT10, while all the other keratinocyte markers were even more drastically downregulated. The key player in melanocyte differentiation, MITF, and the melanogenic enzymes were expressed after transdifferentiation, but only MITF and DCT were significantly upregulated. The expression of MITF was about 300-fold increased and DCT was 25-fold higher expressed (Figure 9B). On protein level expression of MITF and DCT could be confirmed with western blot. Two close but rather weak bands were present for DCT in the control lane where the melanoma cell line HT-144 was loaded. The same bands were present in the lane where the MT-MET-4 protein lysate was loaded. Yet, these bands were clearly stronger. DCT was not detected in the MET-4 protein lysate. The western blot for MITF revealed no clear bands but rather a smear between approximately 40kDa to 80kDa, which could be separated into three broad bands. Different isoforms of MITF have been detected because the antibody used detects melanocyte specific and nonmelanocytic isoforms of MITF. In the control lane the strongest signal was visible around 55kDa (Figure 9C). This is around the actual size of the melanocyte specific MITF-M which is supposed to be detected at 54kDa and the phosphorylated form (pMITF-M) at 60kDa [173]. MITF-M is the smallest isoform of MITF and therefore migrates faster in an SDS-PAGE compared to the bigger isoforms. At these sizes very weak bands are present for MET-4 and stronger bands for MT-MET-4. Thus, MITF is slightly expressed in MET-4 cells and the expression is increased in the transdifferentiated form. Above these bands at around 80kDa a third band is present in MET-4 and MT-MET-4 while only a weak signal is visible for HT-144. This band indicates another non-melanocyte specific isoform, eventually MITF-A, since it is reported to be widely expressed. The other isoforms MITF-C, MITF-D and MITF-H are not expressed in melanoma cells [174, 175]. Additionally, melanocyte-related markers (EDNRB3, PAX3, and PMEL) and the MITF antagonists FOXD3 and SOX2 were tested (Figure 9D). Only a slight increase in expression was noted for PAX3 and EDNRB3 while the expression of PMEL and FOXD3 did not change. Yet, SOX2 expression was significantly reduced in MT-MET-4.

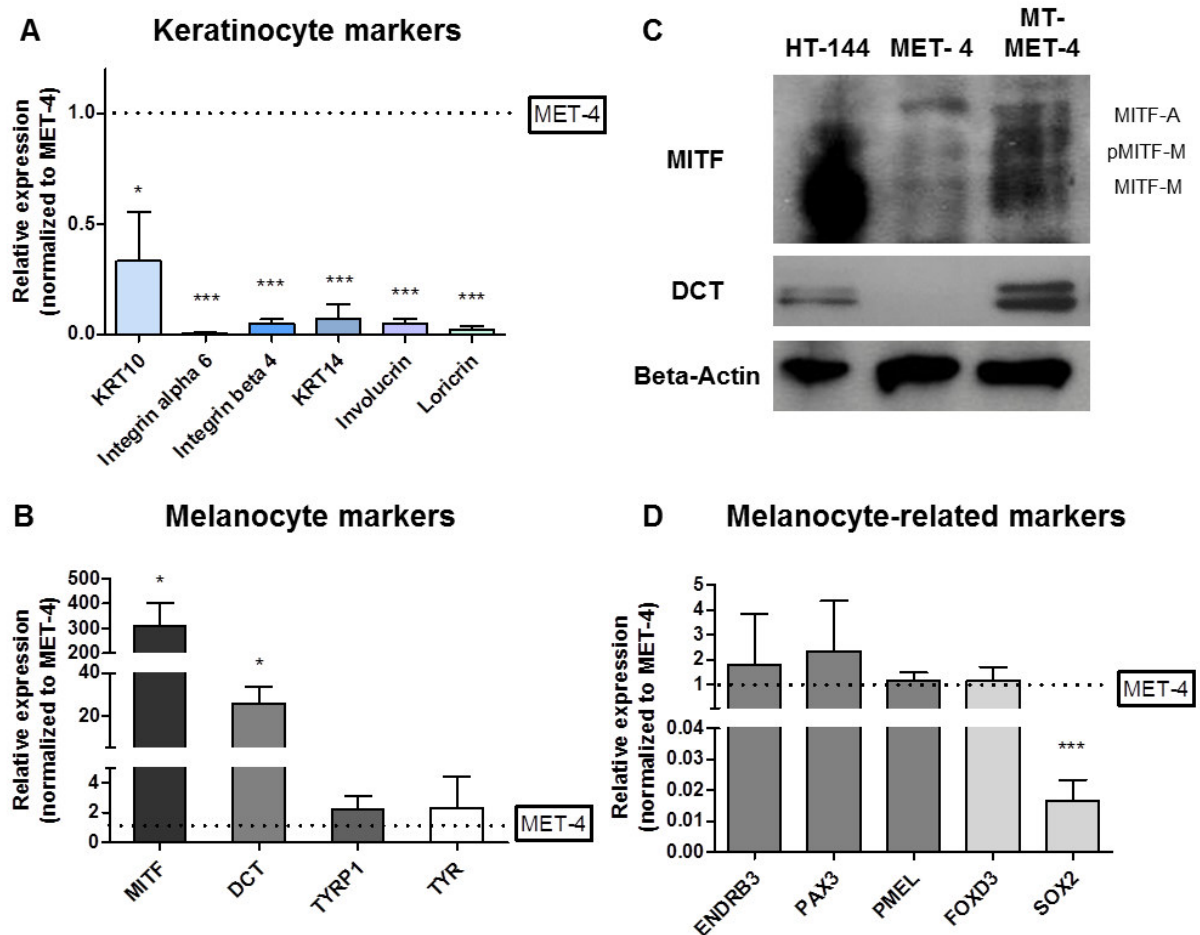


Figure 9: Keratinocyte and melanocyte marker expression. A) All keratinocyte markers tested were significantly downregulated in the MT-MET-4 cells relative to the expression in MET-4 cells. B) The expression of some melanocyte markers was upregulated after transdifferentiation. MITF and DCT were significantly upregulated. C) Western Blot of melanocyte markers MITF and DCT. Expression could be confirmed on protein level D) Expression of melanocyte-related markers such as EDNRB3, PAX3, PMEL, and FOXD3 was not activated. SOX2 was significantly downregulated in MT-MET-4 cells. Error bars indicate the SD of three independent experiments. P values were calculated by two-tailed, unpaired sample t-test. Asterisks indicate t-test p-value < 0.05 (***) p value < 0.005).

One very important and distinct characteristic of melanocytes is the synthesis of melanin, known as melanogenesis and the presence of melanosomes. A whole genome expression array was performed and the resulting data was screened for genes related to melanogenesis. Gene set test in Chipster using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) terms identified differential regulation of several pathways correlated to melanocytes and melanogenesis. The KEGG gene set test revealed that genes correlated to the terms lysosome, tyrosine metabolism, melanoma and melanogenesis were significantly differentially expressed (Figure 10A). Genes related to the terms melanosome, establishment of melanosome localization, melanosome localization,

melanosome transport, melanocyte differentiation were identified *via* analysis using GO. Additionally, eleven terms related to pigment or pigmentation were detected to be differentially regulated (Figure 10B+C).

Gene test enrichment with KEGG performed in Chipster revealed a positive correlation of deregulated genes of MT-MET-4 cells to melanogenesis by showing approximately 80% of the 24 dysregulated genes being upregulated (Figure 10D). This upregulation indicated the activation of melanin synthesis, a hallmark of melanocytes and melanocyte-like cells. Amongst these positively correlated genes various genes related to WNT signaling were present. To these differentially regulated genes belongs one isoform of the WNT-receptor FZD, and two isoforms of TCF, a transcription factor of the WNT signaling pathway. Additionally, the MC1R, a MAPK isoform, two CREB isoforms, and a KIT ligand showed positive correlation to melanogenesis. Negative correlation was shown for the genes EDN1, TCF7, CREB3L2, WNT5A and LEF1.

Electron microscopy revealed the presence of a few melanosome-like structures in MT-MET-4 cells. They could be assigned to stage I to stage III melanosomes (Figure 10E). Small round vesicles with blebs inside, resembling early endosomes, indicated stage I melanosomes. Stage II melanosomes showed fibrils inside the lumen, while in stage III melanin was accumulated inside the vesicle. However, the melanin was not assembled on the fibrils. Thus, these organelles did not seem to contain fibril structures comparable to those seen in eumelanosomes but rather resembled melanosomes containing pheomelanin. In comparison, melanosomes of stage I-IV were observed in melanocytes. These melanosomes were electron dense and contained the typical fibrils (Figure 10F).

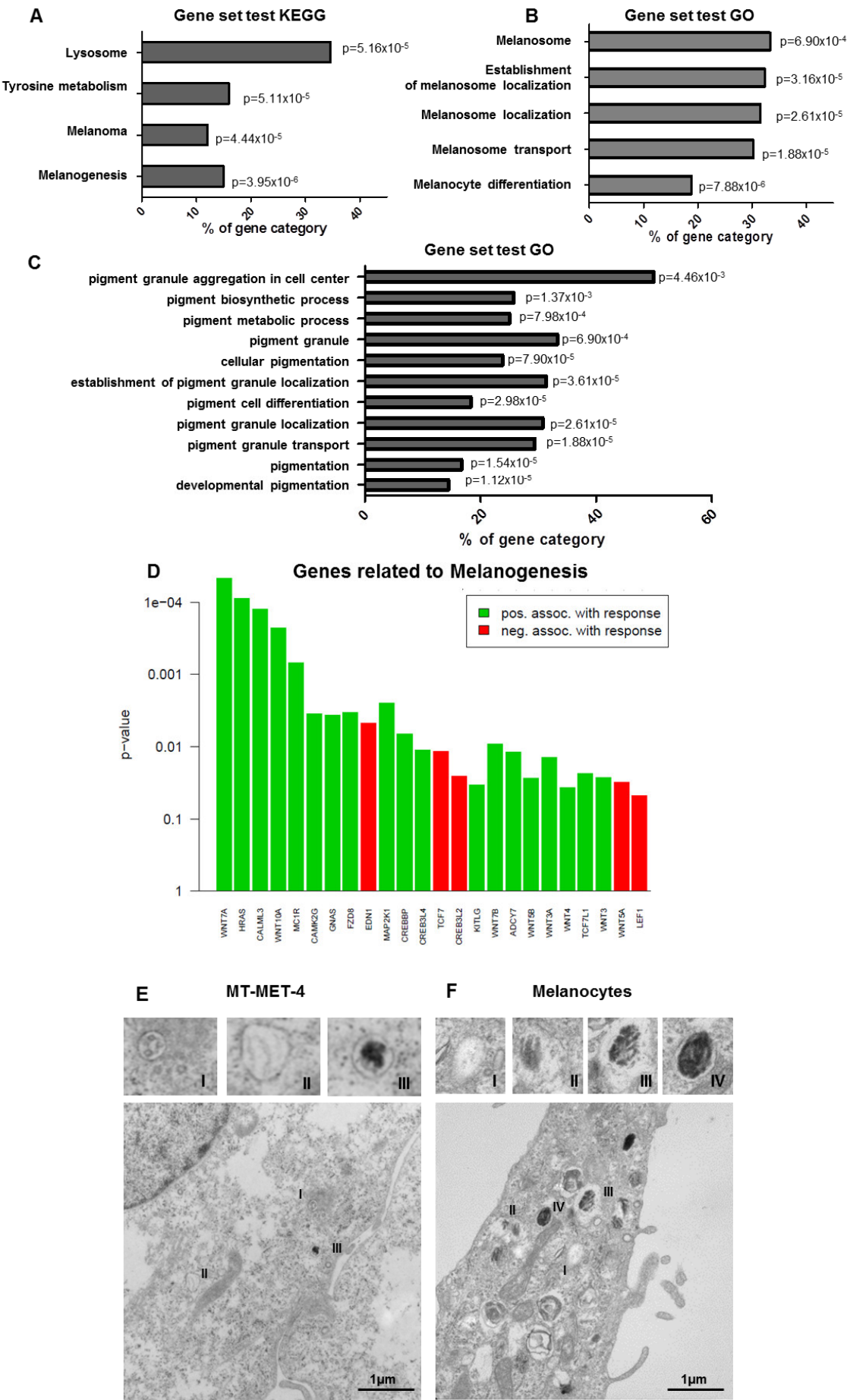


Figure 10: Melanin synthesis in MT-MET-4 cells. A-C) KEGG and GO gene set test showed positive correlation of gene expression in several genes related to melanocyte characteristics such as melanogenesis and pigmentation. D) Several genes related to melanogenesis showed a positive correlation. E) Melanosomes in stage I-III were present in MT-MET-4 cells F) Electron microscopy pictures showing melanosome stage I to IV in melanocytes.

VIII.3. Correlation of MT-MET-4 gene expression to melanoma

Differentiation of cancer cells into another cell type is hypothesized to lead to impaired tumorigenicity [167]. To investigate whether the tumorigenic SCCs were transdifferentiated into normal melanocytes or rather into the malignant form, melanoma cells, the whole genome expression of MET-4 and MT-MET-4 was compared to normal human melanocytes and five melanoma cell lines (Figure 11A). Opposite gene expression patterns were detected for primary melanocytes and melanoma cell lines. MET-4 and MT-MET-4 clustered closely together showing that the general gene expression was still comparable. The majority of the genes in MET-4 and MT-MET-4 showed similar expression pattern to the melanoma cell lines. Thus, a closer correlation between MET-4, MT-MET-4 and melanoma cells was identified compared to melanocytes. Nevertheless, some genes in MET-4 and MT-MET-4 cells revealed an analogical expression pattern to melanocytes. Hierarchical clustering of the whole genome expression data of these samples demonstrated that the gene expression pattern of MT-MET-4 was not altered in a significant manner compared to the parental MET-4 cells. Moreover, a closer correlation between melanoma cell lines and MT-MET-4 cells was detected compared to melanocytes. Thus, according to the whole genome expression analysis MT-MET-4 cells resemble melanoma cells rather than melanocytes.

Gene set test using KEGG identified overall positive correlation of MT-MET-4 gene expression to melanoma. In total, 12 genes were differentially regulated in MT-MET-4 cells compared to MET-4 cells which correlated with melanoma (Figure 11B). Out of these 12 genes eight showed a positive correlation and only four genes were negatively correlated. The genes with positive correlation were HRAS, PTEN, MAP2K1, PIK3CD, BAD, PIK3CA, ARAF and IGF1R. Negative correlation was related to EGFR, MET, PIK3R1 and E2F2.

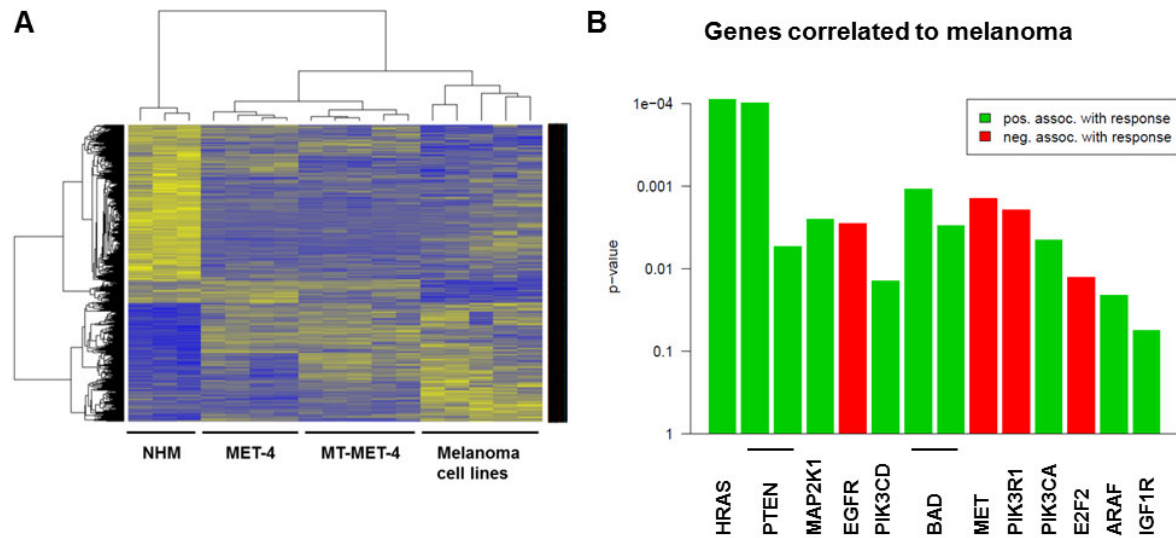


Figure 11: Whole genome expression data. A) Microarray analysis revealed that the expression profile of MT-MET-4 is still comparable to the expression profile of MET-4 if compared with normal human melanocytes (NHM) and melanoma cell lines. The expression profile of MT-MET-4 cells is more similar to the expression profile of melanoma cells than to normal melanocytes. B) KEGG gene set test demonstrated a correlation to melanoma. The gene expression of several genes related to melanoma showed positive correlation.

VIII.4. Minor genomic alterations and changes in methylation after transdifferentiation

Structural changes of the genome are a profound source of genetic variations which has major effects on the phenotype. To exclude the possibility that the observed phenotype is due to genetic changes a single nucleotide polymorphism (SNP) analysis using comparable genome hybridization array (aCGH) was performed to compare the copy number of all sequences of the genomes of MET-4 and MT-MET-4. Amplifications, deletions, copy number changes and copy number neutral loss of heterozygosity (LOH) can be detected via this method. Copy number variation (CNV), thus increase or decrease of chromosomal copies for a given region, was analysed with Genome Studio. Depending on the signal intensity a certain colour code was applied to each sequence. Orange showed hemizygous deletions, green stood for LOH while blue was applied to regions which were amplified and white depicted presence of two copies.

The two metrics for analysing CNV, B Allele Frequency (BAF) and Log R Ratio (LRR) were used. BAF is a normalized representation of the calls for B Allele. A deviation of an average of 0.5 implied CNV. This metric cannot distinguish between LOH and amplification since it is

not a genotype call. For signal intensity the LRR is used. This metric normalizes the signal intensity for CNV analysis. For each region an expected R value (equalling the sum of normalized red and green signal intensity) exists which was calculated based on pre-existing data. The ratio of observed R intensity and expected R intensity is determined for each region. The Log R ratio is calculated as follows: $\text{Log R ratio} = \log_2 (R_{\text{obs}}/R_{\text{exp}})$. A deviation from an average of 0 showed higher or lower signal intensity than expected for 2 chromosomal copies [176].

Overall high amplification was noted for MET-4 and MT-MET4 cells. Such genomic alterations are typical for cancer cells. A closer look at the copy number of the single chromosomes showed that most of the regions of the genome were present in the same copy number however also minor differences in copy number were detected. Three chromosomes, namely 2, 3 and 10 are depicted representatively (Figure 12 A-C). The small arm of chromosome 2, 2p, was amplified in MT-MET-4 cells while it was present at normal copy number in MET-4, indicated by the colours blue and white, respectively. The q arm of chromosome 2 was amplified for both cell types. Only a small sequence was deleted in MET-4 cells. The colour code also differed for parts of chromosome 3, yet by detailed analysis no significant difference in copy number was detected. The signal measure for MT-MET-4 must have exceeded the threshold slightly so that instead of hemizygous deletion LOH was assigned. Chromosome 10 represented various alterations in copy number whereas the changes observed were the same in MET-4 and MT-MET-4. Further the loci of various genes of interest have been investigated. For most of the genes, including MITF, KRT14, ITGA6 as well as P53 and HRAS, no difference in CNV was noted (Figure 12 D-I). If differences were observed they did not relate to the phenotype seen. For example the locus of DCT was amplified in MET-4 cells while normal copy number was present in MT-MET-4 cells. Thus, the increased expression of DCT in MT-MET-4 cells is not due to higher copy number.

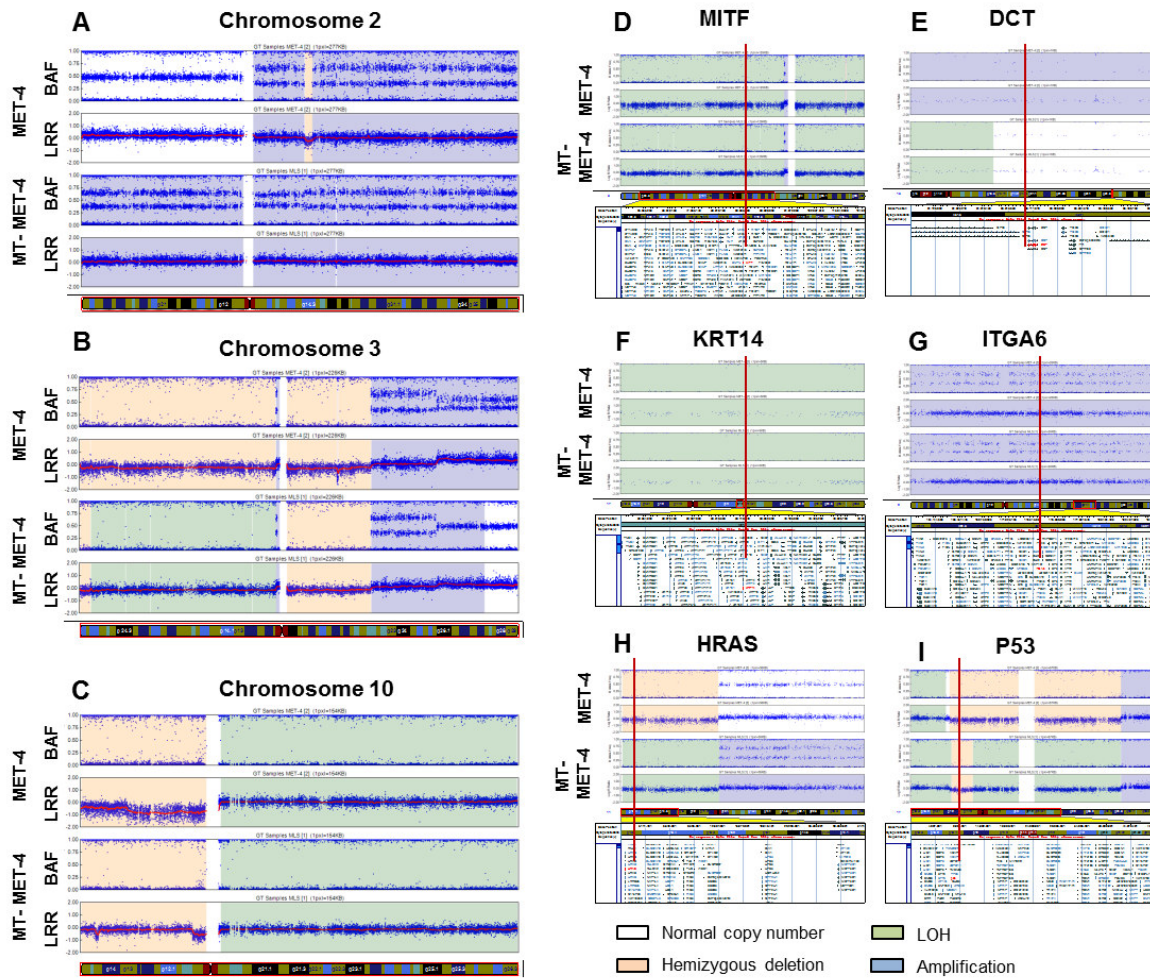


Figure 12: Comparative genome hybridization array (aCGH). A-C) Copy number variation of single chromosomes. Most parts of the genome did not show any copy number variation. Yet, for example the small arm of chromosome 2 is amplified in MT-MET-4 cells while two copy numbers are present in MET-4 cells. Closer analysis revealed that the copy number of some loci was very similar but due to the threshold applied by the analysis software a different color code was assigned as for example seen on chromosome 3. The plot for chromosome 10 depicts high genetic alterations. Yet, they were the same for MET-4 and MT-MET-4. D-I) Copy number at certain gene loci. The loci of genes of interest have been analysed in detail. Most sequences analysed coding for melanocytic (MITF and DCT) or keratinocytic (KRT14 and ITGA6) markers were present in the same copy number. The locus of DCT was amplified in MET-4 cells but was present as two chromosomal copies in MT-MET-4. Additionally no variation in copy number was detected for loci of several tumor suppressors and oncogenes. HRAS and P53 are shown representatively.

In addition to the CNV analysis, two SNP analyses were performed. The genotype of almost 300,000 SNP loci received from aCGH was analysed. Further, the quality control during methylation array analysis included another SNP analysis to identify sample mixup according to the genetic background. A heat map of the SNP probes was created for identification of

eventual sample mixup. The samples with the same genetic background should cluster together.

For the first SNP analysis the genotypes of 293,869 loci were compared. Only 1,978 loci were identified with different genotype. Thus SNP analysis detected variation in the genotype of only 1% of the loci (Figure 13A). The SNP analysis which was included in the quality control could not differentiate MET-4 from MT-MET-4 cells according to the genetic background. The three samples for MT-MET-4 clustered close together. Additionally two samples of MET-4 clustered in close proximity to these three MT-MET-4 samples. These five samples clustered closer together than one of the MET-4 samples to the other two MET-4 samples. Thus, according to the clustering resulting from the SNP analysis MET-4 and MT-MET-4 samples are genetically undistinguishable (Figure 13B). These data show that the cell types still have the same genetic background. In conclusion, only minor genetic changes have occurred during transdifferentiation which were very unlikely to explain the phenotype.

Epigenetic changes including methylation and histone modifications are supposed to occur during the processes of reprogramming and transdifferentiation. Yet, investigation of the methylation status of MET-4 and MT-MET-4 did not demonstrate significant changes. The three MET-4 samples clustered closely together and the MT-MET-4 samples clustered closely together indicating similar methylation pattern within each of the three samples of these cells. The distance between these two cell types, however, was still very close. The dendrogram of quantile normalized clustered samples depicted only very low distance below 0.06 according to Pearson correlation (Figure 13C). In dendrograms the Pearson correlation indicates the distance between different samples which is given in 1- Pearson correlation. The global DNA methylation was clearly increased in the transdifferentiated cells. The methylation of MT-MET-4 was higher in the open sea regions, the islands as well as for the north shelf and north shore and the south shelf and south shore regions (Figure 13D). Thus, the overall methylation in MT-MET-4 samples was higher independent of the region.

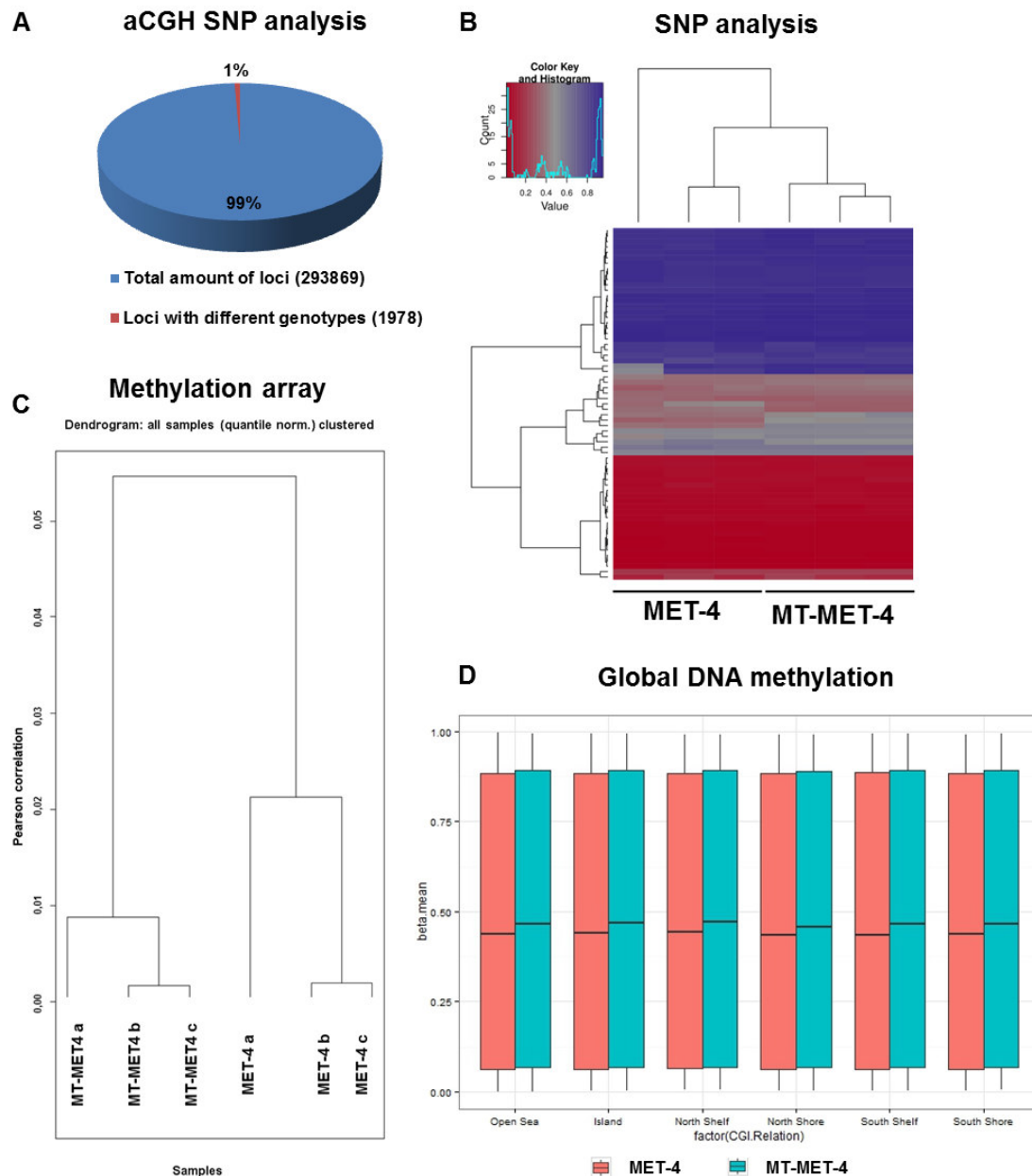


Figure 13: SNP analysis and methylation array analysis. A) aCGH SNP analysis. The genotype of 293,869 loci of MET-4 and MT-MET-4 was compared. Only 1% (1,978 loci) of the loci had a different genotype. B) Methylation array SNP analysis. High similarity was detected between MT-MET-4 cells and MET-4 cells confirming that these two cell types are genomically still very similar and appeared here to be indistinguishable from each other. C) Dendrogram of methylation array analysis. All three MET-4 samples clustered together and all three MT-MET-4 samples clustered together. The distance between the two cell types was very low with a value below 0.06. Pearson correlation in dendrograms depicts the distance (distance = 1 - Pearson correlation) D) Global DNA methylation. General higher methylation was found in MT-MET-4 cells compared to MET-4 at every region of the genome.

VIII.5. MT-MET-4 cells exhibit a reduced malignant phenotype

Different functional analyses were performed in order to measure the presence of cancer hallmarks such as increased proliferation, metabolic activity, apoptosis, and cell motility.

According to their behaviour in culture, the transdifferentiated MT-MET-4 cells seemed to proliferate slower compared to the parental MET-4 cells. A growth analysis was performed to investigate cell proliferation by counting cell number over a period of 16 days. Significant differences in cell growth were measured. The cell number of MET-4 cells increased in a linear way during the whole observation time starting with 50,000 cells and reaching up to 1×10^6 cells. In contrast, MT-MET-4 cells showed strongly decreased proliferation. After day 9 the cell amount reached 2×10^5 which then remained relatively constant with only slight increase until the end of observation (Figure 14A). In addition to the growth curve a BrdU assay was performed as another measurement for cell proliferation. The amount of incorporated BrdU can be determined by measuring the intensity of the coloured reaction product. Its intensity is proportional to the amount BrdU incorporated into the DNA. Cells were incubated for 24h with BrdU before measurement. A significantly higher signal was detected for MET-4 cells, indicating a higher proliferation rate. The absorbance was approximately three fold higher compared to the signal determined for MT-MET-4 (Figure 14B).

The metabolic activity was investigated using an alamarBlue assay with measurements taken at 24h, 72h, and 7d after seeding. After 24h almost the same signal was measured for MET-4 and MT-MET-4 cells. Yet after 72h and 7d in culture significant differences were observed. The increase in signal intensity was more pronounced for MET-4. The relative fluorescence unit (RFU) increased with a factor of 2.28 for MET-4 after 72h while the signal for MT-MET-4 increased approximately 1.76fold. In the final measurement after 7d the signal intensity for MT-MET-4 was not even half as strong as determined for MET-4 cells (Figure 14C). Thus, in addition to reduced proliferation, MT-MET-4 cells showed markedly reduced metabolic activity.

Inactivation of apoptosis signaling, the controlled cell death, can result in tumor initiation, promotion and metastasis and therefore plays a central role in a malignant phenotype [177, 178]. The activity of caspase-3 and -7 was investigated as a measurement for apoptosis. In mammalian cells, the two caspases play crucial roles in programmed cell death. After 3h after addition of the caspase substrate a significantly higher signal was measure for MT-MET-4 cells (Figure 14D). Thus, more active caspase-3 and/or -7 molecules were present.

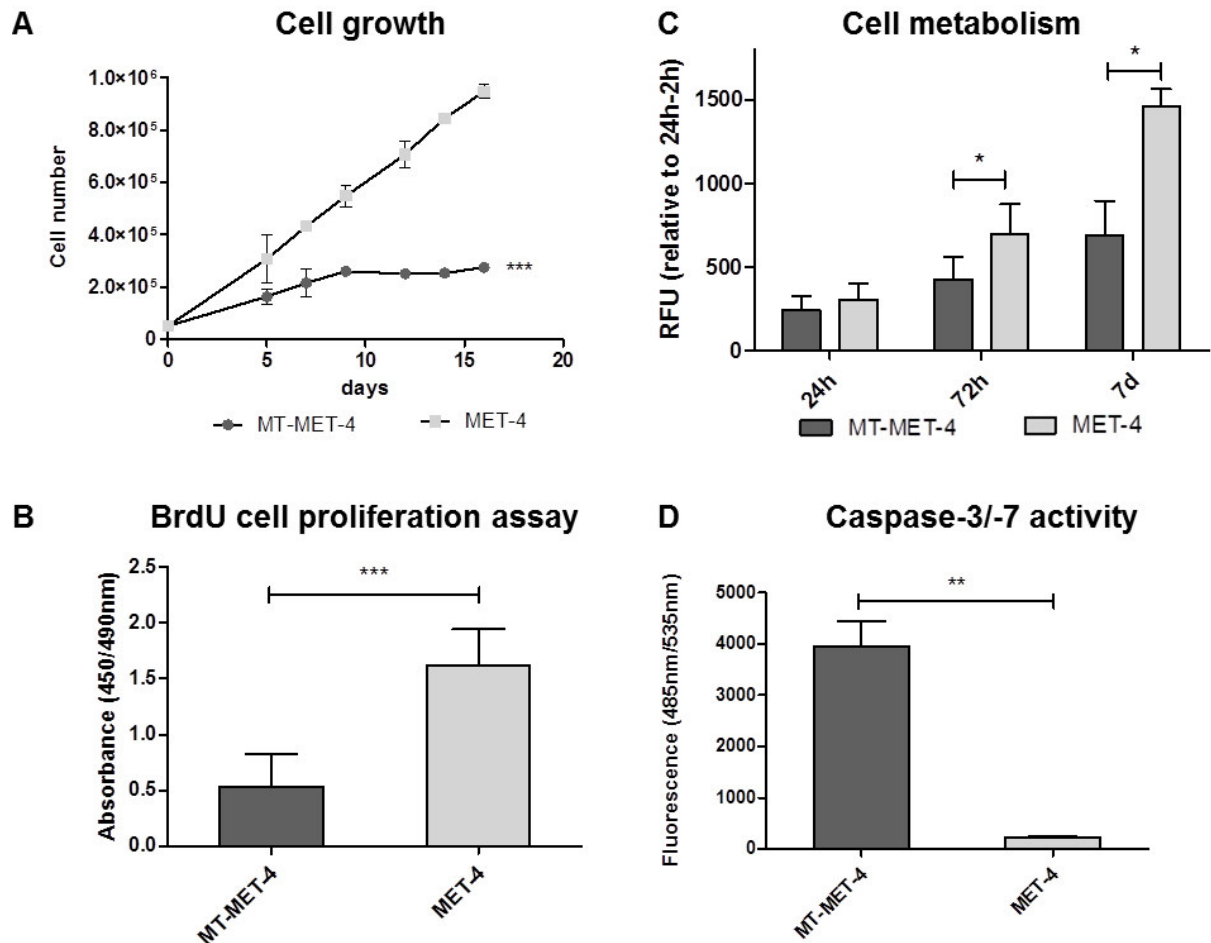


Figure 14: Functional assays: Proliferation, cell metabolism and apoptosis. A) Cell growth analysis. MET-4 cells grew in a linear way during the time of observation while MT-MET-4 cells almost stopped growing after 9 days. Error bars indicate the SD of three independent experiments. P value was calculated by two-way ANOVA, *** p value < 0.005. B) BrdU cell proliferation assay was performed. C) Cell metabolism assay. The metabolic activity of the cells was assessed at three time points (24h, 72h and 7d) after seeding. Statistical significant differences were noted after culture of 72h and 7d. D) Apoptosis assay. The activity of caspase-3/-7 was investigated as a measurement for apoptosis. Error bars indicate the SD of three independent experiments. P values were calculated with two-tailed, paired sample t-test, p-value < 0.05, (**p-value < 0.01, *** p value < 0.005).

Following, the cell movement and cell motility was investigated using a migration and an invasion assay. To study the migration potential of the cells Ibidi chambers were used to guaranty the same migration distance (Figure 15A+B). Cells were observed for 24h while treated with the proliferation inhibitor mitomycin C. Titration of the proliferation inhibitor was necessary because MT-MET-4 cells were more sensitive to the inhibition. The final concentration of proliferation inhibitor used was 1µg/ml for MET-4 cells and 0,125µg/ml for MT-MET-4 cells. MET-4 cells closed 50% of the free area after 10h and almost complete closure was noted after 24h. For MT-MET-4 cells minor reduction of the free area was visible within 10h and after 24h still 80% free area was measured (Figure 15C).

The invasion chamber assay revealed that the cells did not possess high invasive capacity. The chambers were coated with 1x, 0.5x and 0.1x basal membrane extract (BME). After 24h using 1x BME and 0.5x BME almost no cells were able to invade through the matrix. Therefore, invasive capacity was compared after 24h using 0.1xBME. The signal intensity measured for MT-MET-4 was only about 50% of the signal measured for MET-4 indicating a strongly reduced invasive potential of MT-MET-4 cells (Figure 15D).

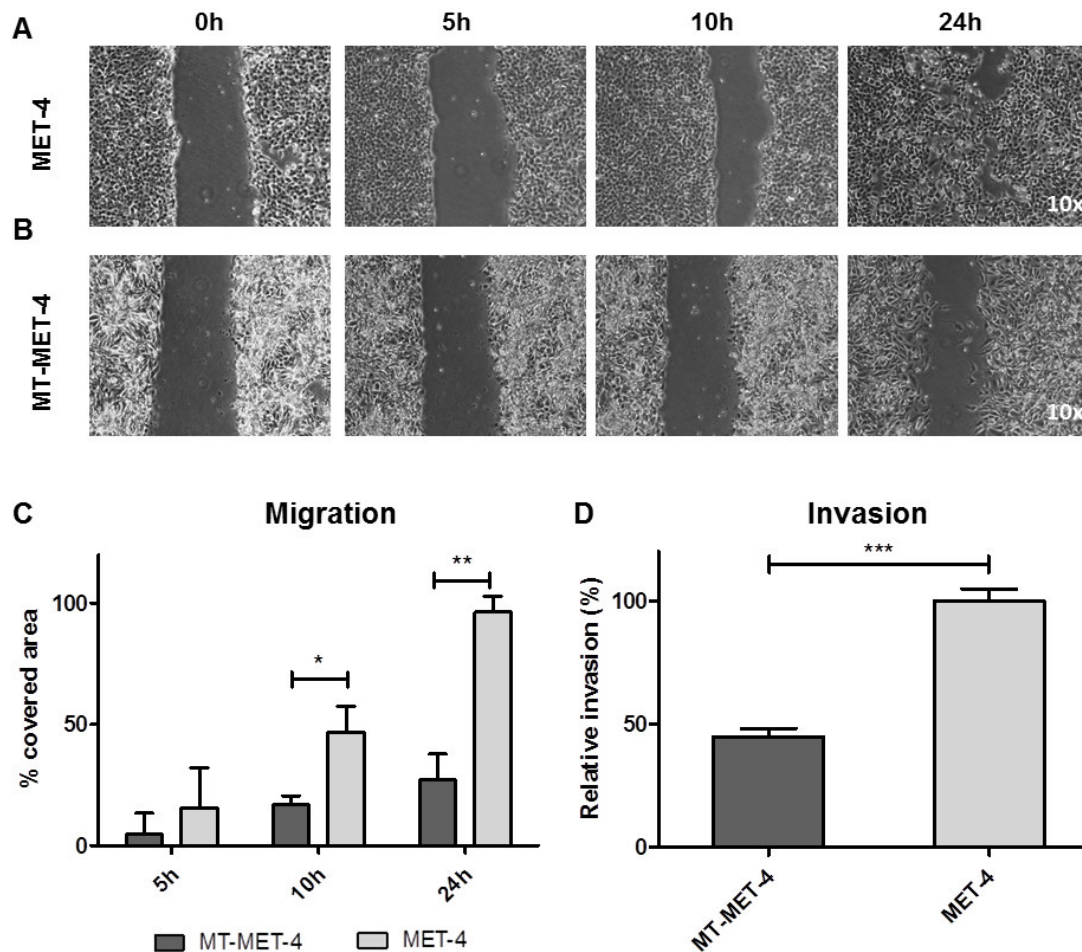


Figure 15: Cell motility. A+B) Migration assay. Culture inserts with a defined cell free gap were used to analyse the migration potential of the cells. After 24h MET-4 cells covered the free area almost completely. The migration potential of MT-MET-4 cells was significantly reduced. C) Quantification of the migration potential. MET-4 cells were able to close more than 90% of the free area within 24h while 80% of the area was still free when using MT-MET-4 cells D) Transwell invasion assay. After 24h the number of invaded cells using 0.1xBME was analysed. The fluorescence signal indicating the amount of invaded cells was 50% lower for MT-MET-4 cells compared to MET-4 cells. Error bars indicate the SD of three independent experiments. P values were calculated by two-tailed, paired sample t-test. Asterisks indicate t-test p-value <0.05 (** p value <0.01, ***p value < 0.005).

VIII.6. MT-MET-4 cells are less sensitive to a MET-4 specific drug cocktail

MET-4 cells are known to have high autophagic activity which also leads to reduced drug sensitivity. To circumvent this drug resistance autophagy inhibition combined with AKT inhibition (AKTi) was used. AKT is known to be involved in the development of skin cancer and therapy resistance. Further, mTOR, the master regulator of autophagy is a substrate for AKT. Inhibition of these two mechanisms in MET-4 cells resulted in increased sensitivity to cisplatin treatment [179]. Combination of autophagy inhibition and AKT inhibition was performed with MET-4 cells and MT-MET-4 cells to ascertain if MT-MET-4 cells are still dependent on autophagy and AKT signaling. After 48h of treatment MET-4 showed strongly reduced metabolic activity (Figure 16A). AKT inhibitor used at 1 μ M reduced the metabolic activity only slightly to about 80% of the metabolic activity measured in the control sample. Single treatment with either autophagy inhibition at both concentrations used or AKT inhibition (10 μ M) had pronounced influence on the viability of the cells. The metabolic activity of MET-4 cells was reduced to 46% for 5mM 3-Methyladenin (3-MA) and to 24% when using 10mM 3-MA. Under the same treatment MT-MET-4 cells showed significant higher metabolic activity of 66% and 52%, respectively. Treatment with 10 μ M AKT inhibitor reduced the metabolic activity of MT-MET-4 cells to 55% while for MET-4 cells 33% was measured. At lower concentrations combination of these two treatments increased this effect. For example, if MET-4 cells were treated with 5mM 3-MA in combination with either 1 μ M or 10 μ M AKT inhibitor the metabolic activity was reduced from 46% to 39% or 26%, respectively. The same observation was made for MT-MET-4 cells. The metabolic activity of 66% after 5mM 3-MA treatment was decreased to 59% and 44%, respectively, if additionally treated with 1 μ M or 10 μ M AKT inhibitor. Also the combination of 1 μ M AKT inhibitor with 5mM or 10mM 3-MA significantly reduced the metabolic activity of the cells. After treatment with 1 μ M AKT inhibitor the viability was reduced to 81% for MET-4 and to 87% for MT-MET-4. Additional 5mM or 10mM 3-MA reduced the metabolic activity to 39% and 23%, respectively for MET-4 while 59% and 44% of the basic metabolic activity was still measured in the MT-MET-4. However, administration of one compound at higher concentrations already led to strong reduction of metabolic activity in MET-4. Addition of another compound only slightly increased this effect. Specifically single treatment with 10mM 3-MA reduced the metabolic activity to 23% which was almost the maximum measured. Addition of 10 μ M AKT inhibitor caused solely another 5% of reduction to ~19% metabolic activity. MT-MET-4 cells were less sensitive to the treatments. Especially single treatments did not have such a pronounced effect. Yet, here the addition of another compound had a stronger effect. In general, MT-MET-4 cells were significantly less sensitive to the drug administration compared to MET-4.

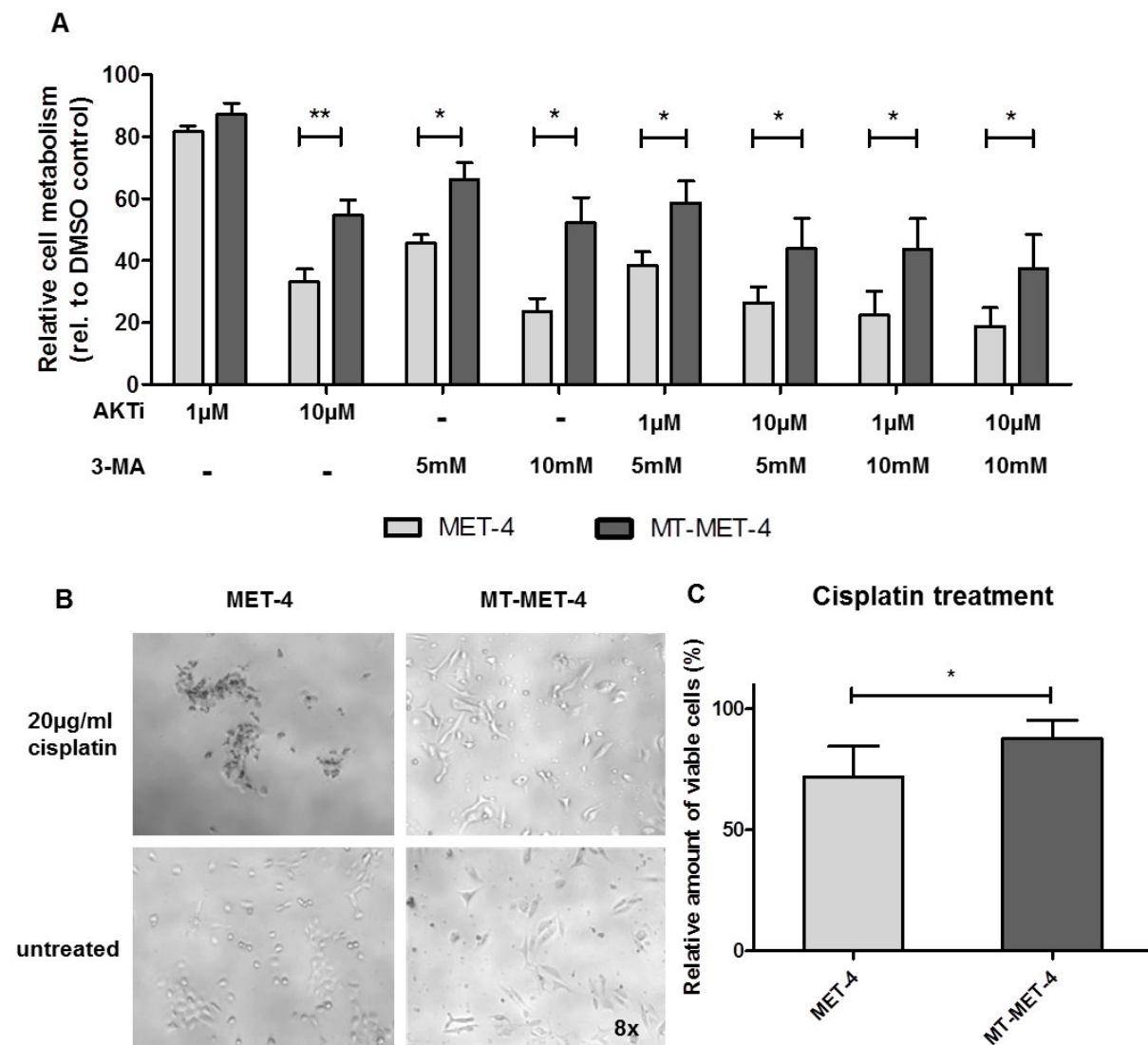


Figure 16: Drug sensitivity assay. A) MET-4 cells and MT-MET-4 cells were treated either with AKTi (1µM or 10µM) or the autophagy inhibitor 3-MA (5mM or 10mM) or with a combination of both compounds. After 48h the viability of the cells was measured. B+C) Cisplatin treatment. Cells were treated with 20µg/ml cisplatin for 48h. Error bars indicate the SD of three independent experiments. P values were calculated by two-tailed, paired sample t-test. Asterisks indicate t-test p-value <0.05 (** p value <0.01).

Additionally MET-4 and MT-Met-4 cells were treated with cisplatin. After treatment with 20µg/ml cisplatin for 24h almost all MET-4 cells seemed to be dead while MT-MET-4 cells did not seem to be strongly affected by the treatment (Figure 16B). After 48h the cell viability was measured using alamarBlue. MET-4 cells showed reduced metabolic activity to approximately 70% compared to the untreated sample while for MT-MET-4 cells almost 90% activity was measured (Figure 16C).

VIII.7. Loss of tumorigenic potential after transdifferentiation

The comparison of the whole genome expression data of MT-MET-4 cells to melanocytes and melanoma cells revealed higher similarity of the gene expression profile of the transdifferentiated cells to melanoma cells rather than to melanocytes. On the other hand, functional assays showed reduced proliferation, cell metabolism, migration, and invasion capacity of MT-MET-4 cells in comparison to the parental MET-4 cells, indicating that the transdifferentiated cells might have a reduced or even impaired tumorigenic potential. To assess the tumorigenic potential of MT-MET-4 cells *in vivo* they were injected subcutaneously into the flanks of NSG mice. Injection of cells into immunodeficient mice is the gold standard to test tumorigenic potential of cells. After 16 weeks first tumor growth was observed for MET-4 cells. In total, tumors grew in four out of four mice within 18 weeks after injection with MET-4 cells. In sharp contrast, no tumor formation within 30 weeks was detected after injection of MT-MET-4 cells (Figure 17A). Tumors isolated from mice which had been injected with MET-4 cells were positive for PAN-CK indicating that these tumors were from keratinocytic origin (Figure 17B).

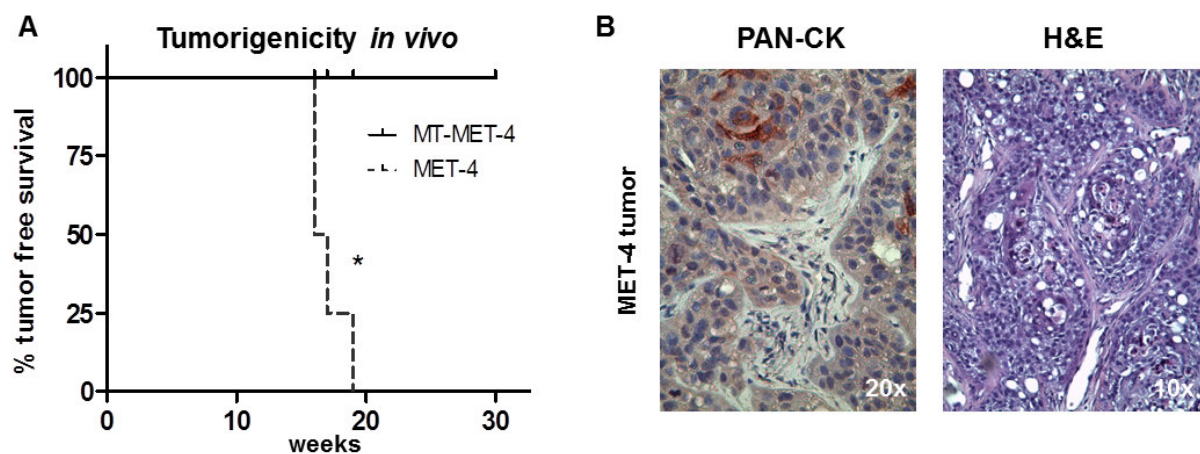


Figure 17: Tumorigenic potential *in vivo*. A) After subcutaneous injection of 1.5×10^6 cells into the flank of NSG mice tumor growth was observed over 30 weeks. For each cell type 4 mice were injected. Tumor formation was observed for all 4 mice injected with MET-4 cells while no tumor growth was detected for MT-MET-4 cells. P-value was calculated with the Fisher's exact test. Asterisks indicate t-test p-value < 0.05 . B) Staining of MET-4 tumor. The tumor which grew after MET-4 injection was PAN-CK positive.

Although the MT-MET-4 cells showed higher similarity in the gene expression profile to melanoma cells than to melanocytes, these cells seemed to have lost their tumorigenic potential. To investigate the mechanism behind this finding whole genome expression data were analysed. IL-24 was the top hit of this analysis with an increased expression of more than 5-fold (Figure 18A). Additionally, a methylation array was performed and the differential methylation between MET-4 and MT-MET-4 was analysed. Here again IL-24 was one of the top hits showing statistically significant differential methylation. Investigation of IL-24 revealed drastically reduced methylation of the promoter regions and the gene body of IL-24 in MT-MET-4 cells. While 76% of the promoter region and 96% of the gene body were methylated in MET-4 cells only 14% of the promoter regions and 32% of the gene body were still methylated in MT-MET-4 cells (Figure 18B). The GSEA of the methylation data combined with the whole genome expression data confirmed the finding that the methylation status of IL-24 in MT-MET-4 cells was correlated to the expression of this gene (Figure 18C). Increased expression of IL-24 could be confirmed with q-PCR. The expression level was approximately 20-fold increased in MT-MET-4 relative to the expression in MET-4 (Figure 18D). Analysis of the CNV of the genomic locus of IL-24 demonstrated that no genetic alteration was present between MET-4 and MT-MET-4 cells. In both cell types the locus of IL-24 was amplified (Figure 18E). Since IL-24 is a cytokine it needs to be secreted to be functionally active [180]. Therefore an IL-24 ELISA was performed. The concentration of IL-24 in the supernatant after 5 days in culture was significantly higher for MT-MET-4 cells compared to MET-4 (Figure 18F). Thus, IL-24 was secreted as a cytokine and might execute its function via a receptor-mediated mechanism. However, the anti-tumor effect of IL-24 is not only related to its functions as a cytokine. It is suggested that intracellular IL-24 promotes the antiproliferative effect seen in tumor cells [181]. Therefore, not only an ELISA with the supernatant but also a western blot with cell lysate was performed. With this method, increased intracellular IL-24 was detected in MT-MET-4 cells (Figure 18G). Ingenuity pathway analysis (IPA) suggested correlation of IL-24 with reduced tumor growth. Specifically, IPA suggested that this gene is involved in reduced proliferation and growth of melanoma (Figure 18H).

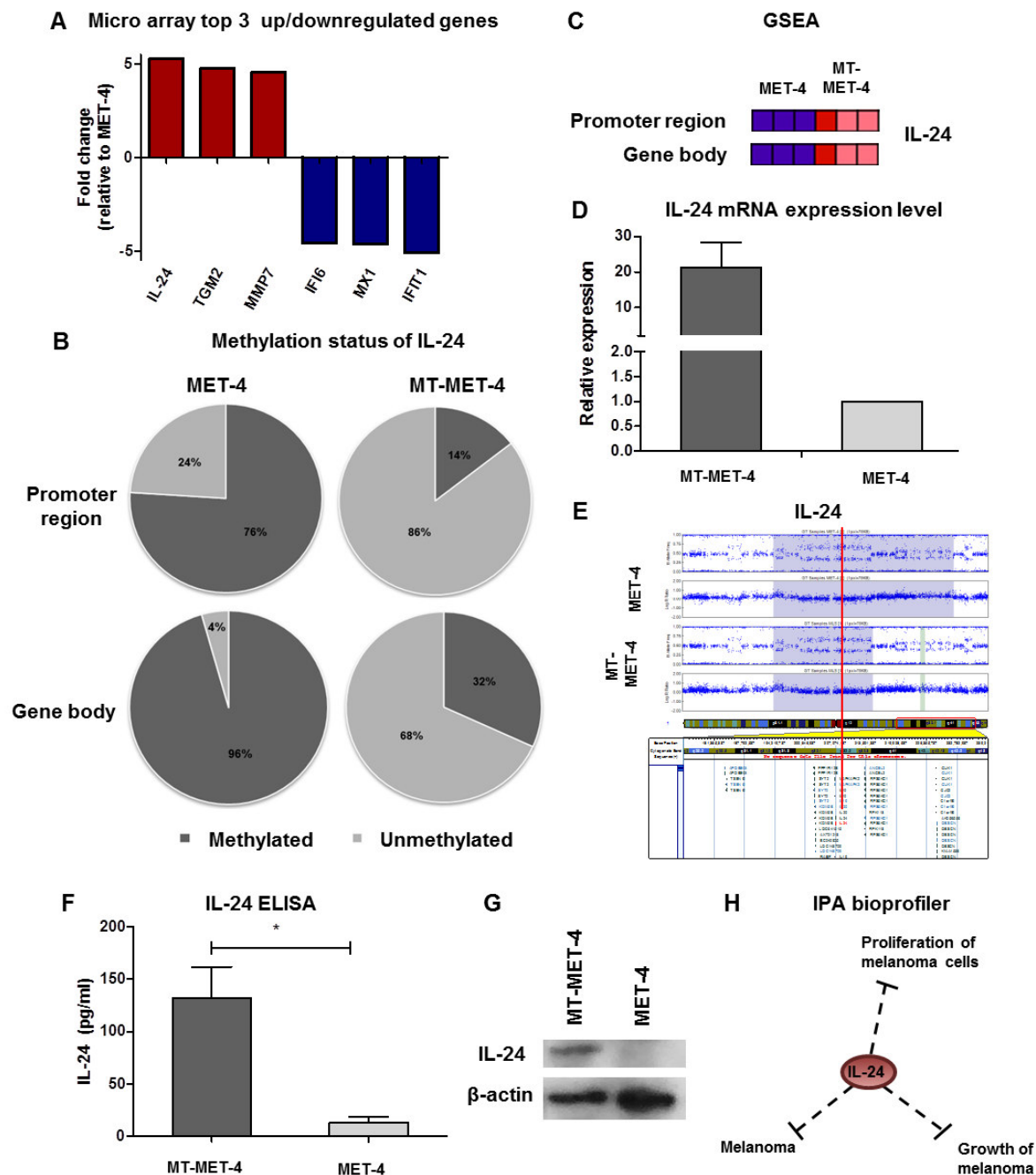


Figure 18: Identification of IL-24. A) IL-24 was the top hit in the whole genome expression array with an upregulation of more than 5 fold. B) Methylation array analysis showed reduced methylation of IL-24 promoter region and gene body in MT-MET-4 cells C) GSEA identified hyper- and hypomethylated genes comparing MET-4 and MT-MET-4 and correlated these findings to whole genome expression data. IL-24 was a top hit after GSEA. D) IL-24 upregulation was confirmed via q-PCR. E) Amplification of the IL-24 locus in MET-4 and MT-MET-4 was detected via aCGH analysis F) IL-24 ELISA. Higher concentration of IL-24 was measured in the supernatant of MT-MET-4 cells compared to MET-4. G) IL-24 Western Blot. Increased intracellular IL-24 protein levels were present. H) Ingenuity pathway analysis bioprofiler for IL-24 and melanoma.

TPA (12-O-tetradecanoylphorbol 13-acetate), one component of the melanocyte transdifferentiation medium, is known to induce IL-24 expression [182]. To exclude that the observed phenotype was caused by TPA MET-4 cells were treated with TPA and TPA was removed from the transdifferentiation medium to investigate the effect on IL-24 expression. MET-4 cells treated with TPA died within 10h after addition of the compound to their normal culture medium DMEM. Probably TPA was the determining factor why MET-4 cells could not be cultured in the transdifferentiation medium. In the transdifferentiation medium without TPA, MET-4 cells could be kept in culture for about a week. The cells did not immediately die, however, they also did not proliferate and thus could still not be cultured in this medium (Supplementary Figure 1). The morphology of MT-MET-4 cells did not change after withdrawal of TPA (Supplementary Figure 2A). Investigation of IL-24 expression in MT-MET-4 cells cultured without TPA revealed solely a slight decrease in expression (Supplementary Figure 2B).

IX. Discussion

IX.1. Melanocytic characteristics and the issues of transdifferentiation

This study shows that cancer cells from the keratinocytic lineage can be transdifferentiated into melanocyte-like cells by ectopic overexpression of the four transcription factors, MITF, LEF1, SOX9 and SOX10. Many keratinocyte markers tested were significantly downregulated indicating loss of keratinocytic characteristics. However, quantitative expression analysis still revealed high levels of integrin beta 4 and moderate expression of p63 and KRT14 in the transdifferentiated MT-MET-4 cells indicating that cells had not fully lost their keratinocytic transcription profile. On the other hand, melanocyte markers like the key transcription factor in melanocyte development, MITF, and DCT, one of the melanogenic enzymes, were significantly upregulated upon induction of the ectopic transcription factors. Analysis of the expression levels of the other two melanogenic enzymes TYR and TYRP1 *via* q-PCR only revealed slight upregulation. Also no other melanocyte-related markers were found to be expressed in the MT-MET-4 cells using q-PCR. Yet, according to the gene set test, several genes related to melanin synthesis and pigmentation were differentially regulated and various genes related to melanogenesis were regulated with positive correlation. SOX2 was significantly downregulated which is in agreement with the literature stating that MITF and SOX2 expression are mutually exclusive and that these genes influence the expression of each other [59]. Additionally, few melanosome-like structures could be detected in the transdifferentiated cells. The phenotype was stable and was maintained after doxycycline withdrawal. Thus, the effect seemed to be independent of transgene expression. Taken together, these findings indicate that the cells adapted some melanocytic characteristics, however probably did not fully transdifferentiate into the melanogenic lineage. Whole genome expression array analysis clearly showed that the transcriptional profile of MT-MET-4 cells still resembled the one of MET-4 cells to a great extent. Remarkably, the transdifferentiated cells, although not fully converted to the melanocytic lineage, did not possess tumorigenic potential anymore.

Another explanation for the low melanocyte marker expression in SCCs upon induction of ectopic expression of transdifferentiation factors might be the presence of a heterogeneous population. Only a minor fraction of the cells had changed their morphology and adapted a complete melanocyte-like phenotype. Therefore, selection of the transdifferentiated cells was necessary. Here, serial trypsinization was used to enrich the transdifferentiated cells. This was possible since the melanocyte-like cells detached faster from the cell culture flask than

the parental MET-4 cells. This is a typical procedure for isolation of melanocytes from mixed cultures which also contain fibroblasts and keratinocytes. Treatment of the cells with G418 is another possible method since melanocytes are less sensitive to this antibiotic [183] than other cell types. However, the MET-4 cells were resistant to the treatment with G418. Thus, this selection method could not be utilized to remove the cells which did not transdifferentiate in order to create a homogenous cell population of melanocyte-like cells. In this study, a melanocyte-specific reporter construct was used to identify the cells which endogenously expressed MITF. GFP expression served as an indicator for the activation of this gene. It was attempted to sort GFP positive cells *via* fluorescence activated cell sorting (FACS), however the cells did not survive this treatment and therefore could not be taken into culture afterwards again. Above that GFP expression was diminished after a certain time in culture. It was not anymore possible to distinguish between the cells which had activated endogenous MITF expression and which had not. Selection according to melanocyte specific surface markers would have been an alternative option. Yet, no reliable surface marker was expressed in the transdifferentiated cells which could have been used.

In order to promote transdifferentiation of SCCs, a defined medium was used that contained several growth factors which are known to promote melanocyte growth and differentiation. This medium contained, among others, basic fibroblast growth factor (bFGF), insulin, hydrocortisone, bovine pituitary extract (bPE), forskolin and 12-O-tetradecanoylphorbol 13-acetate (TPA). The long-term proliferation of melanocytes is stimulated by bFGF [184]. Insulin, forskolin and bPE promote the growth of melanocytes [185, 186]. Additionally, bPE enhances the dendricity of these cells [187]. Hydrocortisone also stimulates the proliferation and differentiation of melanocytes [188]. TPA is known to enhance dendricity and proliferation of melanocytes and to lead to inhibition of apoptosis [189, 190]. Forskolin directly activates the adenylyl cyclase and increases the intracellular cAMP concentration [191] leading to activation of MITF transcription and induction of the melanogenic signaling network. Keratinocytes express bFGF to stimulate melanocyte proliferation and melanogenesis. Additionally, endothelin is expressed by keratinocytes which stimulates mitogenesis and melanin synthesis in melanocytes [192].

Normal human melanocytes also need a cocktail of various cytokines and growth factors in the culture medium to be able to grow. It is known that melanocytes are dependent on the cytokine secretion of keratinocytes for proliferation and differentiation [193, 194]. Therefore, these cells cannot be passaged over a long period in culture especially without addition of sufficient growth stimulating factors. Also for the differentiation of ES or iPS cells differentiation media contained several additional factors to guide the cells into the melanocytic lineage [161, 164]. Interestingly, Nissan and colleagues succeeded in

differentiating iPS cells into melanocytes with only addition of BMP-4. However, after differentiation into melanocytes, the cells were seeded in medium containing human melanocyte growth factor supplements [164].

In the present study a strong effect of the medium on the phenotype of the SCCs was observed. The parental MET-4 cells could not be cultured in the melanocyte transdifferentiation medium. Only cells which underwent transdifferentiation were able to grow under these conditions. Yet, if transdifferentiated cells were cultivated in the medium used for the parental cells the phenotype quickly switched back and they lost the melanocytic morphology. TPA was identified to be the main factor causing death of MET-4 cells when cultivated in the transdifferentiation medium. TPA is a known tumor promoter which was used to eliminate fibroblasts and keratinocytes from mixed cultures with melanocytes [195]. Since MET-4 cells are from keratinocytic origin they might be sensitive to this factor. Above that TPA induced expression of IL-24. This cytokine is an antitumor gene which selectively kills tumor cells by apoptosis induction but does not harm 'normal' cells. This might also be an explanation why MET-4 cells died after addition of TPA.

The genomic status can have enormous influence on the transdifferentiation ability of the cells. The p53 induced DNA damage response reduces the reprogramming efficiency. Often cancer cells carry p53 mutations which makes cells more amenable to transdifferentiation. This process is a stressful condition under which cells normally activate p53 and undergo apoptosis. If p53 is not present, the cells cannot activate this escape mechanism. In the literature it is stated that temporary suppression of p53 enhances the reprogramming efficiency [196]. Often, p53 is mutated in skin cancer cells from sun exposed sites. Additionally, the RAS genes, specifically HRAS, are commonly mutated in skin cancer cells. The genomic aberrations of MET-4 cells have been well characterized. These cells are wild type for p53 and all RAS genes [197]. Moreover, no genomic alterations were detected in the MT-MET-4 cells. This shows that no subclass of MET-4 cells with altered p53 or RAS loci was selected during transdifferentiation. Thus, this is not an explanation why MET-4 cells were at least partly responsive to transdifferentiation.

Melanin synthesis is a distinct characteristic of melanocytes. To test if this process was activated in the transdifferentiated cells a gene set test was performed. Several terms correlated with pigmentation and melanosomes were differentially regulated. Additionally, several genes related to melanogenesis were differentially regulated leading to a positive correlation. Both findings indicated that melanin synthesis might have been induced. Presence of melanosomes can be demonstrated via electron microscopy. Melanosome-like structures were detected in the MT-MET-4 cells. However, only few of these vesicles were discovered. Beyond that, these structures did not resemble typical eumelanosomes which

have a fish bone-like structure and are elongated. The vesicles found in MT-MET-4 cells were spherical and did not show this intraluminal fibrillary organization. The electron dense parts, which were probably melanin, were condensed at certain areas inside the vesicle and not evenly distributed. This appearance resembles rather the one of pheomelanosomes, which contain the less abundant red or yellow melanin or immature eumelanosomes. Several factors are known to affect melanin synthesis and influence the type of melanin. Factors which have an effect on this process are for example tyrosinase activity, melanocortin 1 receptor (MC1R) variants, as well as the concentration of cAMP and sulfur compounds [71].

Melanogenesis is a well-studied and in detail characterized process. The synthesis of melanin takes place in three distinct steps. First cysteinylDOPA (CD) isomers are formed until the cysteine concentration drops below 0.13 μM . Following pheomelanin is produced by oxidation of these CD isomers. This stage continues until the CD concentration falls below 9 μM . Finally, the production of eumelanin is initiated. This only starts after most of the CD isomers and cysteine is exhausted. Thus, tyrosinase activity, tyrosine and cysteine availability determine the ratio of eumelanin to pheomelanin [198].

Upregulation of tyrosinase expression was noted after transgene expression in MET-4 cells. However, after doxycycline withdrawal only low tyrosinase levels were measured in MT-MET-4 cells on mRNA level. Low activity of the key enzyme in melanin synthesis is correlated with production of pheomelanin. Tyrosinase is responsible for the production of DOPAquinone. With low tyrosinase activity only few DOPA molecules are generated. The thiol compounds exceed the concentration of DOPA which leads to conjugation of DOPA with the thiol compounds. The DOPA molecules are then being trapped favoring pheomelanin synthesis [71].

Binding of α -melanotropin to MC1R can stimulate the synthesis of eumelanin. However, the gene encoding this receptor is known to be highly polymorphic influencing the type of melanin. Distinct allelic variants are correlated to increased pheomelanin and skin cancer. Specific polymorphisms lead to loss of function and reduced response to melanotropin. Non-functional MC1R has a significant influence on cAMP level and pigmentation since binding of α -melanotropin to the receptor normally stimulates cAMP production and tyrosinase activity in a dose-dependent manner. Specifically combinations of different alleles of MC1R are associated with this change in function. Melanocytes with these polymorphisms are more sensitive to UV radiation and resulting DNA damage. This probably explains the association of certain receptor variants to increased skin cancer risk [199].

PMEL is responsible for the formation of the fibrillary structures inside the eumelanosomes [82]. Since this gene was not expressed in the MT-MET-4 cells, it might also be possible that

actually eumelanosomes are present in these cells, yet the structural protein is missing which gives rise to the typical shape and morphology.

Successful transdifferentiation of one cell type into another depends on several factors, including transcription factor combination and method used to introduce these factors into the cells as well as cell type of origin and medium composition, which decisively determines the outcome and efficiency of this process. There is a multitude of explanations for low efficiency or incomplete transdifferentiation. Firstly, in this work, multiple transcription factors were introduced into MET-4 cells with the help of lentiviral particles, which harboured one factor each. Because of that, it was not guaranteed that all cells were infected with each factor or with the same ratio of factors. The result of a lentiviral vector infection is a heterogeneous population of cells that express different combinations of all the factors used at different levels. Wang and colleagues demonstrated that the stoichiometry of transdifferentiation factor expression levels correlates with the efficiency of transdifferentiation [200]. The concentration of transgene expression in the cell can also have crucial influence on the efficiency. Low expression might be sufficient to reduce the marker expression of the original cell line but cannot induce the transcriptional program of the cell type of interest. Additionally, transgene silencing can also affect the expression level and duration of transgene expression and thus reduce transdifferentiation efficiency. Since lentiviral vectors randomly integrate into the host genome, their integration might damage genomic sites which are essential for survival or proper functioning of the cell. Further cells can be refractory and do not manage to overcome transcriptional or epigenetic barriers to induce transdifferentiation [142, 167].

Also the cell type of origin significantly determines the capacity and efficiency to transdifferentiate. The transdifferentiation of HaCaT cells, a cell line established from spontaneously immortalized keratinocytes from human skin, with MyoD1 to induce myogenic differentiation took longer than conversion of other cell types. Additionally, several keratinocyte differentiation markers were still expressed after transdifferentiation. After treatment with 5-aza-2'-deoxycytidine myogenic marker expression was increased but keratinocyte markers were unaffected [201].

Cells from the keratinocytic lineage seem to be more committed to their origin and more difficult to convert into other cell types. Wernig and colleagues noted very low transdifferentiation efficiency when transfecting keratinocytes with factors for induction of neuronal cell fate. They concluded that it is probably due to the chromatin state of keratinocytes at the binding sites of ASCL and BRN2 [202].

Above that the cell fate conversion of human cells in general is more complex than for murine cells. Cells from human origin seem to be more resistant to reprogramming towards pluripotency induced by ectopic expression of transcription factors [142]. Similar observations were reported for transdifferentiation of human cells [166]. Murine fibroblasts adapted melanocyte characteristics by only ectopically overexpressing MITF [165]. These cells adapted melanocyte morphology, expressed melanogenic markers and contained electron dense vesicles resembling immature melanosomes. In the MET-4 cells MITF alone did not have such an effect. MITF could be expressed ectopically in MET-4 cells; however, the activation of endogenous MITF transcription was not observed. Cells which only expressed MITF could also not survive in the melanocyte medium which should favour transdifferentiation.

According to Yang and colleagues, the transdifferentiation of murine fibroblasts into melanocytes was faster than conversion of human fibroblasts. After 5 days of transcription factor induction in mouse embryonic fibroblasts (MEFs), melanocyte markers were expressed. The infected cells were cultured in the presence of G418 for 14 days to enrich melanocyte-like cells. Similar results were obtained with mouse tail fibroblasts. The efficiency of transdifferentiating human fibroblasts was much lower and it took longer to generate almost pure melanocyte populations. 40 days after infection of the human cells and selection with G418 only about 40% of the cells were positive for TYR and TYRP1. Human cells needed to be cultured for 100 days to gain 99.3% TYR positive cells [166].

Sometimes also additional factors are necessary to generate the same cell type from human cells compared to murine cells. For example, murine fibroblasts were converted into neurons by expression of only three factors, *Ascl1*, *Brn2* (also called *Pou3f2*) and *Myt1l* [123]. The addition of *NEUROD1* was necessary to generate neurons from human fibroblasts [203].

The factor combination identified in this study might not be sufficient to fully activate the endogenous transcription profile of melanocytic cells. Fibroblasts from murine and human origin were successfully transdifferentiated into functional melanocytes using the factors MITF, SOX10 and PAX3 [166]. Yang and colleagues used mouse fibroblasts which express GFP after endogenous activation of TYR. With this method first of all it was demonstrated that ectopic overexpression of SOX10 and MITF suffices to induce transdifferentiation towards the melanocytic lineage. Upon addition of a third factor highest amount of GFP positive cells with 10.06% was measured when PAX3 was added. The combination of SOX10, MITF and LEF1 yielded only 4.04% of GFP positive cells while SOX9 increased the percentage from 6.44% up to 9.53%. Thus, SOX9 also seemed to play an important role in the induction of the melanocytic transcription profile. The four factor combination SOX10, MITF, PAX3 and SOX9 only increased the amount of GFP positive cells from 10.06% to

10.71%. The combination of MITF, SOX10 and PAX3 proved also effective in converting human fibroblasts to melanocytes. Considering the conclusions from this study, it might be that PAX3 was missing among the candidate factors used for transdifferentiating SCCs into fully functional melanocytes. PAX3 is described as a key player in melanocyte development [30, 58]. Important to consider is that at least 8 isoforms of PAX3 exist and different isoforms have distinct effects on melanocyte development [29, 33]. Yang and colleagues used PAX3e which seemed to be the relevant isoform. In contrast to this, the isoforms PAX3a, b, c, d, and i were among the candidate transcription factors used in our study. None of these PAX3 isoforms favoured the transdifferentiation of SCCs into melanocytes.

According to the observations made here SOX10 seemed to be essential for the transdifferentiation of SCCs into melanocytes. Only cells which had been infected with SOX10 showed high endogenous MITF expression. SOX10 might bind early to the DNA in this process thereby making the chromatin accessible for the other transcription factors, thus having 'pioneer factor' activity. Yet, binding of a single SOX10 protein to genomic loci does not alter transcription. Binding partners are necessary to elicit this function [204]. Possibly, SOX10 is essential to serve as a binding partner for the other transcription factors. During melanocyte development SOX10 and PAX3 form a complex to activate MITF. Thereupon, SOX10 and MITF cooperate to induce DCT and TYR expression [31, 45, 60].

Another SOX family member, SOX2, has been described as pioneer factor for certain genes in ESCs [205]. The role of SOX2 in melanocyte development is not completely clear. Adameyko and colleagues described a mutually exclusive expression of SOX2 and MITF. Further, SOX2 and MITF showed cross-regulatory effects in the development of melanocytes in the chick embryo. SOX2 repressed MITF expression and MITF suppressed SOX2 expression [59]. In contrast, Cimadamore and colleagues suggest that SOX2 is essential for endogenous expression of MITF in hESC-derived neural crest (hESC-NC) cells and normal human melanocytes *in vitro* [206]. In the present study, SOX2 was also downregulated upon transdifferentiation and upregulation of endogenous MITF expression supporting the cross-regulatory effect of these two molecules.

Mestre-Escorihuela proposed that due to accumulation of DNA methylation and chromosomal rearrangement, cell lines might eventually be more difficult to transdifferentiate [207]. It was demonstrated that higher methylation was present in primary cell lines compared to the tissue from which these cells have been isolated from. Increase in methylation has been noted specifically in regions close to transcription start site [208]. Additionally, changes in DNA methylation of trophoblast cell lines were suggested to be induced during long term cultivation of these cells. The analysis of the methylation profile of cancer cell lines compared to primary tumors revealed that cancer cell lines in general

contain increased levels of CpG island hypermethylation than cells from primary tumors [209].

The chromatin state is a determining factor for accessibility of DNA binding sites for transcription factors. Open chromatin structures are necessary for most transcription factors to be able to bind and to initiate gene expression [210]. By analysing the chromatin state at certain loci which are known binding sites for the transcription factors used for transdifferentiation it is possible to estimate the responsiveness and efficiency of this process. Wernig and colleagues analysed the interaction of the three transcription factors ASCL1, BRN2 and MYT1L. Thereby, they identified a unique trivalent chromatin signature which was essential for ASCL1 binding [202]. Presence of this signature was predictive for the transdifferentiation of several cell types into neurons. For example, high enrichment of this trivalent chromatin state was present in human fibroblasts, while only low enrichment was noted in human keratinocytes. Resulting ASCL1 binding to the predicted sites was high in fibroblasts but very low in keratinocytes. Fibroblasts and other cells with enriched chromatin signature could be transdifferentiated into neurons. However, Wernig and colleagues were not successful to transdifferentiate keratinocytes into neurons with the same transcription factors. Additionally, they suggest a distinct hierarchy amongst these three factors. ASCL1 acted as a pioneer factor, binding first to the certain genomic sites and then recruiting BRN2 to these loci.

Therefore, it would be of interest to analyse the chromatin state of the binding sites of SOX10 and MITF to better understand the process of transdifferentiation into the melanocytic lineage and also study the interaction of these factors. Possibly, using this method, an additional cell line could be identified which can be transdifferentiated into melanocyte-like cells using the protocol established in this work.

The differentiation state seems to play an important role in the amenability of cells to reprogramming and transdifferentiation [211]. The expression levels of lineage markers can be a hint for the differentiation status. Yet, the analysis of keratinocyte markers was not able to predict the transdifferentiation efficiency in SCC cell lines. Only in MET-4 cells, thus in one out of seven SCC cell lines, melanogenic characteristics could be induced by ectopic expression of the transcription factor combination. MET-4 cells, which are derived from a metastatic tumor [197], expressed low amounts of keratinocytic markers. The differentiation state of cancer cells often correlated with the progression of the disease. Tumor cells from metastasis are therefore often poorly differentiated [212]. However, some metastases especially from carcinomas are known to be well differentiated, sometimes even showing higher degree of differentiation compared to the primary tumor [213]. Since MET-4 cells are derived from a carcinoma it is difficult to draw a conclusion on the actual differentiation state

and if due to a low degree of differentiation these cells were more responsive to transdifferentiation.

To increase the transdifferentiation efficiency OCT4 could be added to the cocktail. OCT4 was identified to increase cell plasticity. It was shown, that OCT4 can lead to dedifferentiation of cells and global demethylation [214, 215]. The chromatin structures were opened, favouring reprogramming and transdifferentiation. Already the transient expression of OCT4 is sufficient to make fibroblasts more amenable to transdifferentiation into early hematopoietic and neural progenitor cells using distinct media [214]. In addition, keratinocytes could be transdifferentiated into neuronal and contractile mesenchymal cell types by transient OCT4 expression [216].

Epigenetic modulation can facilitate reprogramming and transdifferentiation events. Treatment of cells with demethylating agents such as 5-azacytidine enhanced the efficiency of cell fate conversion. For keratinocytes it has been shown that OCT4 expression led to global demethylation facilitating reprogramming of these cells [216, 217]. Following, Bickenbach and colleagues found out that decitabine treatment activated OCT4 transcription [218]. Later they discovered that single application of decitabine and culture in specific neuronal medium was sufficient to induce neuronal markers. Thus, epigenetic modifications have a pronounced influence on the cell plasticity and reprogramming efficiency. [219].

C-MYC is known to enhance the transcription of genes, which stimulate proliferation. Therefore, c-MYC was first thought to promote proliferation during somatic reprogramming [220]. However, the function of c-MYC as a transcription factor has been reconsidered. It was shown that c-MYC does not activate a certain set of target genes but rather amplifies the transcription at every active site in general [221, 222]. Considering these findings, expression of c-MYC should be able to favour any cell fate conversion event [220]. Thus, addition of c-MYC to the transcription factor cocktail might stimulate the expression of melanogenic markers and promote transdifferentiation into the melanocytic lineage.

IX.2. Genetics versus epigenetics in transdifferentiation and tumor formation

Cancer cells are usually genomically unstable and continually accumulate genomic aberrations (reviewed in [223]). To exclude that the melanocytic phenotype and the impaired tumorigenic potential observed in the MT-MET-4 cells resulted from genomic alterations aCGH analysis was performed. This analysis revealed that few copy number variations (CNVs) were present. Yet, the majority of the genome showed exactly the same signal for corresponding sequences in MET-4 and MT-MET-4. Additionally, SNP analysis showed that

only 1% of the analysed loci varied in sequence. Thus, it is highly unlikely that the phenotype is caused by genomic alterations.

Epigenetic alterations are known to take place during differentiation, reprogramming and are also suspected to happen during transformation of cells into tumor cells [128]. Promoter hypermethylation, which was found to be associated with CpG rich regions, is a prominent epigenetic alteration commonly found in several cancer cells. Especially the promoters of tumor suppressor genes were often strongly methylated leading to reduced or even suppressed expression [224]. This modification was identified to favour tumorigenesis. Therefore, suppression or removal of methylation was thought to be a possible cancer treatment. The demethylating agents 5-azacytidine (5-aza-C) or decitabin were used to treat cancer in the hope that this reactivates the expression of tumor suppressor genes which then counteract cellular transformation [225, 226]. In addition to promoter hypermethylation, global hypomethylation was discovered in cancer cells. It was identified as an early event in the transformation process while the extent of hypomethylation strongly depends on the type of cancer and the origin of the tumor sample [227-229]. Metastases seemed to be even more susceptible to global hypomethylation compared to cancer cells from the primary tumor [230]. Cells from cancer metastases are often less differentiated which might be related to the significant overall demethylation of the genome. The global hypomethylation increases genomic instability and can lead to activation of genes related to tumor promotion. Additionally, the interaction of euchromatin and heterochromatin is altered which also stimulates tumor formation [229]. Thus, the use of demethylating agents such as 5-aza-C might not always be beneficial but rather harmful.

In this study, a methylation array was performed to investigate and compare the epigenetic landscapes of MET-4 and MT-MET-4 cells. The two different cell types could clearly be distinguished from each other, yet, overall only minor changes were observed. Since MET-4 cells are of metastatic origin probably reduced methylation might be present in these cells in general. Global methylation analysis demonstrated increased methylation at every locus in MT-MET-4 cells indicating a more differentiated state. However, also the promoter regions were stronger methylated in MT-MET-4 cells. This analysis only shows the relative global methylation status in MT-MET-4 cells in comparison to the parental MET-4 cells. For this reason, it is not possible to distinguish whether the promoters in the MT-MET-4 cells were indeed hypermethylated or if the low methylation rate in MET-4 cells created the impression of a promoter hypermethylation in MT-MET-4 cells.

Ballestar and colleagues compared the methylation status of pre B-cells and macrophages, which had been transdifferentiated from pre B cells. A high throughput methylation analysis of these two cell types detected no significant changes in the promoter regions of cell type-

specific marker genes. Yet, histone marks had changed according to the altered gene transcription. Active histone modifications (H3K9/K14 acetylation and K4 trimethylation) were found at the promoter regions of macrophage-specific genes near the transcription start site in transdifferentiated cells and correlated with the activation of macrophage-associated genes. Additionally, H3K27 trimethylation (H3K27me3) was reduced at these sites. In accordance with this, the activating histone marks were decreased at B-cell-related genes while the suppressive marks did not increase in B cells during transdifferentiation. These findings suggest a hierarchy in epigenetic alterations. As a conclusion, histone regulators seemed to be more potent and could overcome repressive DNA methylation [231].

In another transdifferentiation study the importance of histone marks was highlighted in addition. The modification of histones in a sequential manner seemed to be essential for stable transdifferentiation of hindgut cells into motor neurons in *Caenorhabditis elegans* (*C. elegans*). The demethylase JMJD-3.1, which specifically removes di- and trimethylation marks at lysine 27 of histone 3 (H3K27) and the methyltransferase Set1, which methylates histone 3 at lysine 4 (H3K4) were identified to play crucial roles in this process. Stepwise modifications of the histones by these two enzymes are necessary for a robust transdifferentiation event. It was suggested that Set1 and JMJD-3.1 play highly conserved roles to strengthen transcription factor induced changes in cell identity [136]. In our study, no significant differences in methylation at the loci of melanocyte and keratinocyte markers were noted. It would therefore be interesting to analyse the histone marks at these sites. According to the above mentioned studies it is likely that histone marks play a more important role in transdifferentiation and therefore rather influence genes expression and not methylation.

These studies show important differences between transdifferentiation and reprogramming according to the epigenetic changes. While during transdifferentiation no major alterations in methylation were observed, the demethylation of promoter DNA is an important event for generation of pluripotent cells *via* reprogramming. Especially the promoters of NANOG and OCT4 need to be demethylated to establish a pluripotent state similar to ES cells [232]. Yet, besides these prominent demethylation events, total methylation landscapes vary significantly between ES and iPS cells. The epigenetic signature of somatic cell of origin was still present in the reprogrammed cells [233, 234]. Thus, a complete adaption of the methylation pattern is not necessary for significant changes in cell characteristics. These data confirm our findings. Significant changes in cell characteristics such as morphological changes and altered functional behaviour was observed while no major changes in methylation could be detected. According to these studies and our findings the methylation

landscape is not a reliable measure for cell fate conversion whereas histone marks should be taken into consideration instead.

IX.3. Identification of IL-24 and its relation to the observed phenotype

After microarray and methylation array analyses IL-24 was identified as a top hit. IL-24 showed the strongest upregulation and its promoter as well as the gene body was highly demethylated. Upregulation of IL-24 after transdifferentiation into the melanogenic lineage might be a sign for adoption of the melanocytic phenotype and differentiation of the cells. In normal human melanocytes IL-24 is highly expressed. Its expression is still present in early stages of melanoma development but becomes downregulated during cancer progression and cannot be detected in advanced melanoma anymore [235, 236]. The mda-7/IL-24 gene was discovered upon differentiation of melanoma cells which had been treated with interferon and mezerein [237]. This induced differentiation treatment leads to an increase in IL-24 expression. Therefore, IL-24 was first associated with terminal differentiation of melanoma cells.

Several factors, which were present in the medium used for cultivating the transdifferentiating cells, stimulate melanocyte differentiation and might be involved in the upregulation of IL-24 in MT-MET-4 cells. TPA for example is known to induce IL-24 expression and was found to increase promoter activity of IL-24 [182]. It was noted that TPA was involved in the upregulation of IL-24 in MT-MET-4 cells since there was a slight reduction of IL-24 expression after TPA withdrawal. However, one can hypothesize that TPA is not alone responsible for the expression of IL-24. MT-MET-4 cells were not dependent on TPA since they also grew in medium without TPA and IL-24 was still expressed.

To exclude that TPA alone was responsible for the induction of IL-24 MET-4 cells were treated with TPA and TPA was removed from the transdifferentiation medium. MET-4 cells died within 10h after addition of TPA to the medium. Removal of TPA resulted in slightly decreased expression of IL-24 but did not completely abolish its expression. Thus, it could be concluded that stimulated differentiation of MT-MET-4 cells resulted in IL-24 expression and TPA only increased its expression. However, it is not fully clear if transdifferentiation into another lineage alone was sufficient for the loss of tumorigenic potential.

Various groups discovered that the treatment with IL-24 recombinant protein or ectopic expression of IL-24 with an adenovirus vector led to growth arrest and induction of apoptosis in cancer cells while IL-24 did not harm 'normal' cells [237]. Therefore, IL-24 was categorized as an anticancer gene which selectively induces cytotoxicity in cancer cells but not non-

malignant cells. IL-24 does not belong to the tumor suppressor genes since its knock out did not lead to tumor formation [238].

IL-24 is involved in several mechanisms and can activate several signaling pathways which promote antitumor effects that are independent of the status of the tumor suppressors p53 and RB [237]. Various apoptotic mediators, such as BAX, BAK, TRAIL, p53, and FAS, as well as several signaling molecules including PKR p38, PI3K, JNK and GSK-3 have been observed to be upregulated upon IL-24 expression [181, 239]. Depending on the cancer type different molecules are activated, however, it seems that all these molecules share a common cell death mechanism resulting in caspase activation and mitochondrial destruction. IL-24 especially induces cleavage of caspase-3 and caspase-9 [240]. Yet, the mechanism of action strongly depends on the cell or tissue type [181, 239]. For example, IL-24 induced apoptosis and growth arrest in G2/M phase in lung cancer cells with a parallel increase in p53, BAX and BAK [240].

In melanoma however, expression of p38 and the resulting overexpression of GADD proteins was observed to be essential for activation of the cell death pathway [241].

The mechanism by which IL-24 exerts its effect depends on its cellular location. IL-24 can act intracellularly but is also secreted. If IL-24 is secreted it functions as a cytokine and binds to its receptors IL-20R1/IL-20R2 or IL-22R1/IL20R2. Receptor binding activates the JAK/STAT signaling pathway resulting in activation of STAT1 and STAT3 [242]. This activation can lead to apoptosis in cancer cells but also receptor-mediated STAT-independent apoptosis was reported [243]. In the immune system IL-24 receptor binding is suggested to activate the secretion of cytokines including TNF- α , IFN- γ and IL-6 [244, 245].

In addition to its induction of cytotoxicity IL-24 possesses an antimetastatic activity by reducing the migration potential and the invasive capacity of the cells. Significant inhibition of migration and invasion was reported after expression of IL-24 *in vitro* [246, 247]. The downregulation of matrix metalloproteinases (MMP-2 and -9), p85 PI3K and pFAK were responsible for this effect. No correlation with the cytotoxic effect of IL-24 was observed. Reduced number of lung metastases were noted when lung cancer cells had been treated with adenoviral IL-24 before intravenous injection into nude mice [248].

Since IL-24 has anticancer functions it might also be possible that due to its expression the cancer phenotype of MT-MET-4 cells was diminished. The effect of IL-24 on melanoma cells is very well described. Therefore, one likely explanation is that IL-24 had a significant influence on the tumor phenotype upon transdifferentiation.

The SCCs could have been transdifferentiated into the melanogenic lineage while retaining the tumor forming capacity. The tumorigenic phenotype is correlated with specific characteristics. Malignant tumor cells mostly show high proliferation, are highly invasive, and resistant to apoptosis. It was observed that melanoma cells are either highly proliferative but less invasive or the other way round [249]. Transformation into non-tumorigenic cells should negatively affect these characteristics. MT-MET-4 cells proliferated much slower, were less motile, and had significantly reduced invasive capacity. Additionally, apoptosis seemed to be upregulated indicated by increased caspase-3/-7 activity. The functional characterization of MT-MET-4 cells led to the conclusion that these cells do not resemble melanoma cells according to their behaviour. Thus, the functional assays performed comparing MET-4 and MT-MET-4 altogether provided indications that the transdifferentiated cells are less aggressive and might have reduced or lost tumorigenic potential.

MET-4 cells are known to have high autophagic activity. They probably activated this process to increase survival. This is a typical mechanism of cancer cells to become more resistant to apoptosis and withstand stressful conditions [250]. Treatment of MET-4 cells with autophagy inhibitor and AKT inhibitor increased the sensitivity of these cells to cisplatin treatment. Cisplatin activates caspase-3 and stimulates apoptosis. By blocking autophagy the cells cannot use their typical escape mechanism and are more responsive to this treatment [179]. The transdifferentiated cells were less sensitive to the MET-4 specific treatment with the autophagy inhibitor 3-MA and AKT inhibition. This indicates that the MT-MET-4 cells might have undergone physiological changes, which render them less dependent on autophagy for survival.

The study of Carette and co-workers supports the hypothesis that cell fate conversion can influence the responsiveness to a specific treatment. Reprogramming of chronic myeloid leukemia cells which were dependent on the signaling of the fusion oncogene BCR-ABL (break-point cluster - abelson), made them lose this dependency thereby becoming resistant to the BCR-ABL inhibitor imatinib. They concluded that imatinib targets a specific population of chronic myeloid leukemia cells with a distinct epigenetic profile. Thus, the epigenetic differentiation state can influence the functionality of a compound or better the responsiveness of a cell to a certain treatment [251].

Another explanation for the impaired tumorigenic potential might give the upregulation of IL-24. It was shown that ectopic IL-24 expression in melanoma cells resulted in cytotoxicity. Inhibition of autophagy however reduced the cytotoxic effect of IL-24 on these cells. Thus, the reduced sensitivity of MT-MET-4 cells to autophagy inhibition might be due to reduced cytotoxic effect of IL-24. The original study additionally treated MET-4 cells with cisplatin. MT-MET-4 cells might be more sensitive to this combination since Yacoub and colleagues could

show that IL-24 expression enhances the toxicity of cisplatin treatment [252]. IL-24 and cisplatin activate the procaspase-3 by cleavage and both activate JNK1/2 leading to cell toxicity. Additionally, cisplatin activates CD95, which was increased in the presence of IL-24. The toxic effect of radiation is also increase by IL-24 [253]. More autophagic vesicles were produced and increased ceramide and ROS signaling was noted. Again, inhibition of autophagy reduced this toxic effect induced by IL-24 and radiation. Yet, still a significant level of cell death was noted. Comparison of cisplatin treatment of MET-4 and MT-MET-4 demonstrated reduced sensitivity of MT-MET-4 cells. This was probably due to the dramatically reduced proliferation of MT-MET-4 cells since chemotherapeutic drugs, such as cisplatin, target dividing cells [254, 255].

Injection of the cells into NSG mice confirmed the hypothesis that transdifferentiation of cancer cells induces a non-tumorigenic phenotype. While in all mice which had been injected with MET-4 cells tumor formation was observed no tumor growth was seen for MT-MET-4 cells. Reduction of tumorigenicity by direct conversion was first shown by Graf and colleagues who converted B cell leukemia and lymphoma cell lines into macrophages. After transdifferentiation with ectopically overexpressing C/EBP α , the cells adapted macrophage characteristics and showed reduced tumor forming potential after injection into immunosuppressed mice [167].

IX.4. Transdifferentiation versus terminal differentiation

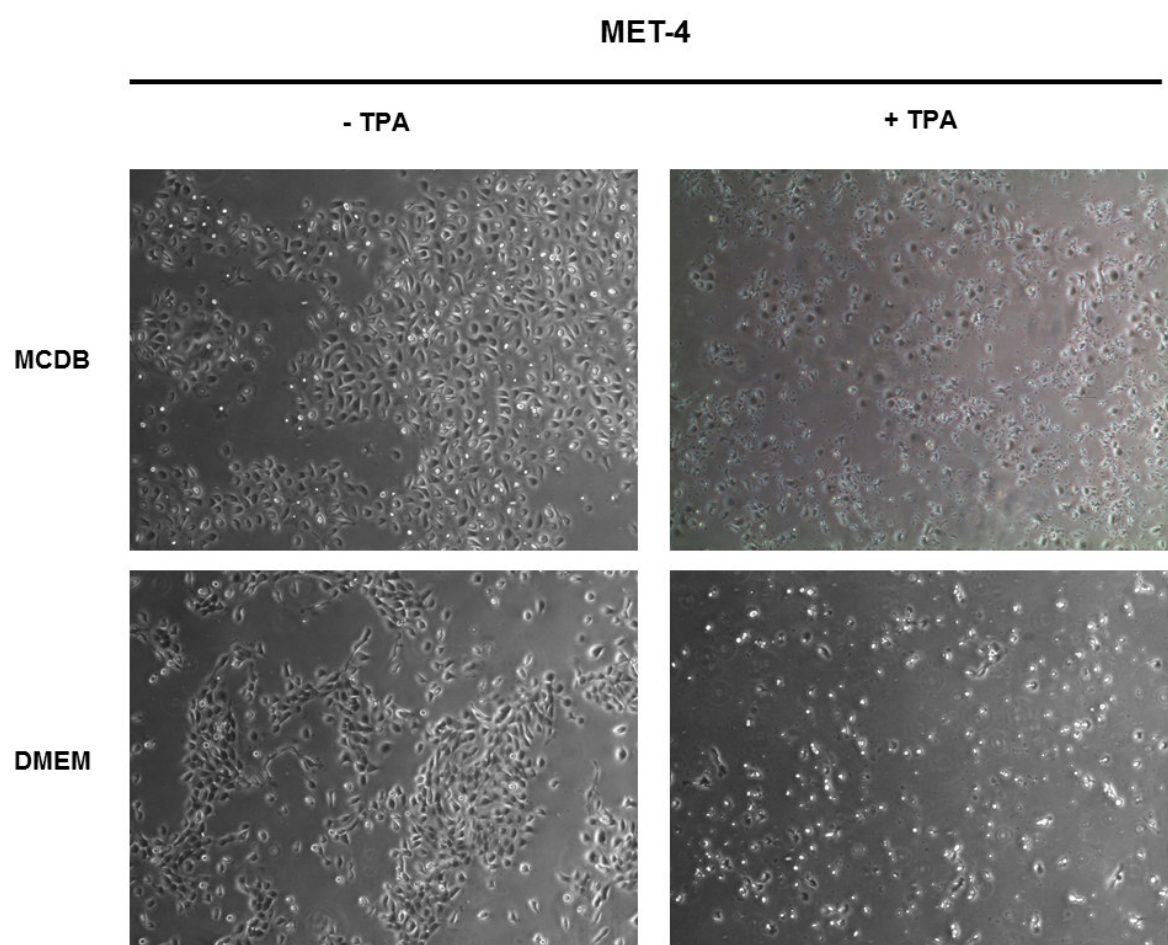
Apart from transdifferentiation, terminal differentiation is a common approach to reduce or impair the tumorigenicity of cancer cells [168, 256]. It is believed that tumor cells activate mechanisms, which impair normal differentiation [257]. Forced upregulation of differentiation-promoting factors could induce differentiation of cancer cells and lead to reduced growth and normalized signaling in these cells. One example for differentiation therapy is treatment of cancer cells with IL-24. This cytokine is used for differentiation of melanoma cells which leads to growth arrest in these cells [258].

Another hypothesis is that cancer cells block their natural differentiation program of their lineage of origin. Thus, for example brain tumor cells cannot be forced to differentiate into normal neurons. Yet, forced differentiation into another lineage should be possible. Additionally, reprogramming of cancer cells should release this block and make cells responsive to differentiation into cells of the same lineage but also of other lineages while impairing their tumorigenicity. Zhang and colleagues asked how far back a cell must be dedifferentiated to be able to differentiate into another lineage. With their study they showed that partial reprogramming of cancer cells is already sufficient to reverse the differentiation block and generate terminally differentiated cells from various lineages [168].

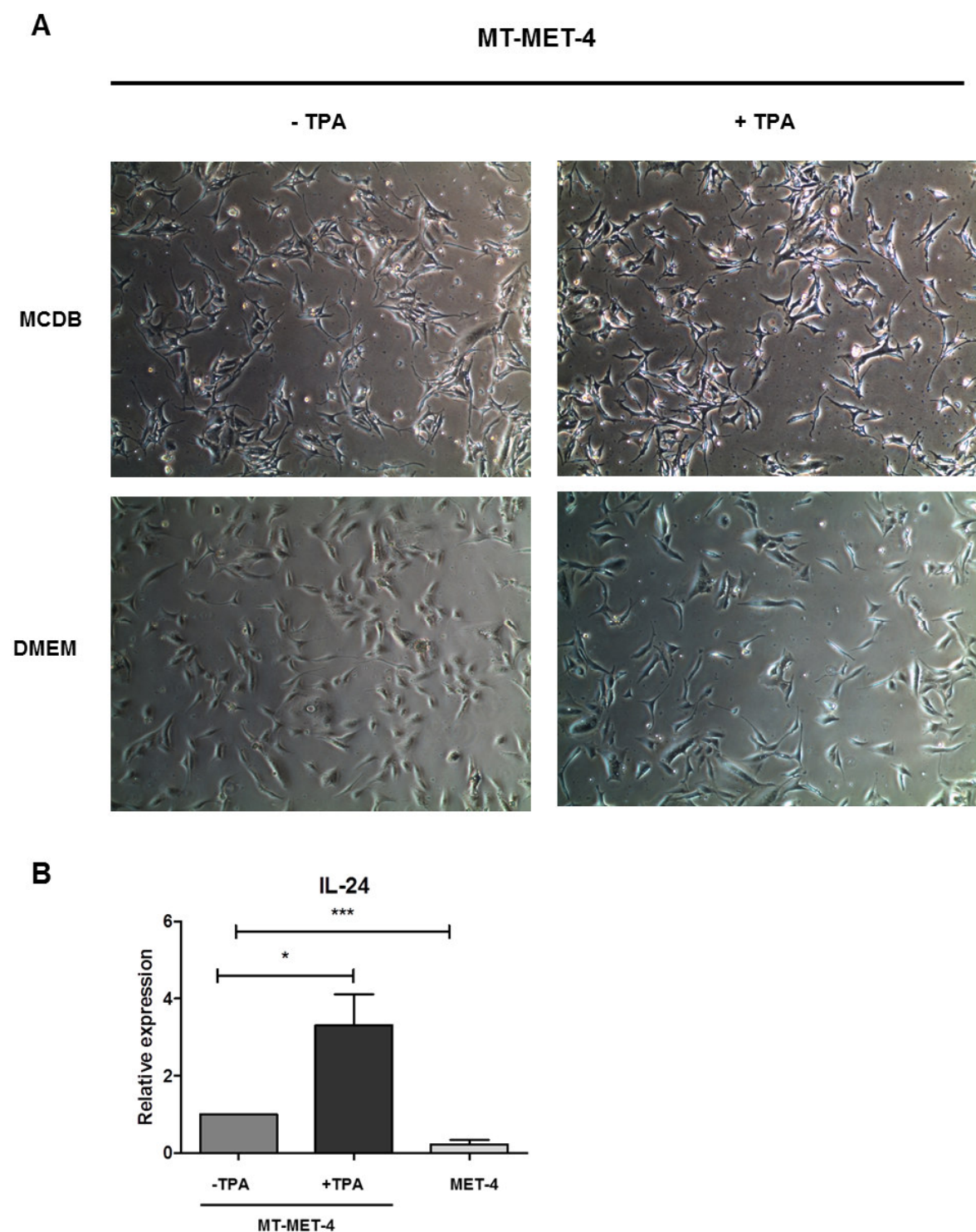
Thus, it might be that due to transdifferentiation into another lineage the MET-4 cells gained their differentiation potential and adapted a more differentiated phenotype which resulted in lost tumorigenicity.

In summary, this study shows that squamous cell carcinoma cells can be transdifferentiated into melanocyte-like cells using lentiviral vectors for ectopic overexpression of a defined set of transcription factors. Additionally, it is a proof of principle that *via* transdifferentiation the tumorigenic potential of cancer cells can be impaired.

X. Supplementary Figures



Supplementary Figure 1: Effect of TPA on MET-4 cells. In medium containing TPA, Met-4 cells die within 24h. If MET-4 cells are seeded in MCDB medium without TPA cells do not immediately die. Yet, they lose their cell-cell attachment and seem to become senescent.



Supplementary Figure 2: Effect of TPA on MT-MET-4 cells and IL-24 expression. A) TPA does not have a strong effect on the morphology of MT-MET-4 cells. The culture medium influences the appearance of the cells strongly. B) TPA increases the expression of IL-24 in MT-MET-4 cells. MT-MET-4 cells express IL-24 also if they are culture in MCDB medium without TPA. MET-4 cells express only very low levels of IL-24.

XI. References

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XII. Abbreviations

°C – Degree centigrade

% - Percentage

α –Alpha

β- Beta

μ - Micro

3D – Three dimensional

3-MA - 3-Methyladenine

5-aza-C - 5-azacytidine

7-AAD - 7-aminoactinomycin D

aCGH - Comparative genome hybridization array

AIM1- Absent in melanoma 1

AKT - Protein kinase B

AKTi – AKT inhibitor

APS - Ammonium persulfate solution

Arg151Cys - Arginine to cysteine change at position 151

Arg160Trp - Arginine to tryptophan change at position 160

ASCL1 - Achaete-scute homolog 1

Asp294 - Aspartic acid at position 294

Asp294His - Aspartate to histidine change at position 294

BAF - B allele frequency

BAK - Bcl-2 homologous antagonist/killer

BAX - Bcl-2-associated X

Abbreviations

BCA - Bicinchoninic acid

BCC - Basal squamous cell carcinoma

BCL2 - B-cell lymphoma 2

bFGF - Basic fibroblast growth factor

BH – Benjamini-Hochberg

bHLH-LZ - Basic-helix–loop–helix-leucine-zipper

BME - Basal membrane extract

BMP - Bone morphogenic protein

bPE - Bovine pituitary extract

BrdU - Bromdesoxyuridin

BRN2 - Murine brain-2

BSA - Bovine serum albumin

C. elegans - Caenorhabditis elegans

CaCl₂ - Calciumchlorid

cAMP - Cyclic adenosine monophosphate

Caspase - CysteinyI-aspartate specific protease

CD19 - Cluster of differentiation 19

cDNA - Complementary DNA

CEBP - CCAAT-enhancer-binding protein

c-KIT - Tyrosine kinase receptor

cm - Centimeter

CMV - Cytomegalovirus

CNV - Copy number variation

CpG - "—C—phosphate—G—", cytosine and guanine separated by only one phosphate

CT - Cholera toxin

Abbreviations

DCT - Dopachrome tautomerase

DHI - 5,6-dihydroxyindole

DHICA - 5,6-dihydroxyindol-2-carboxylat

DLX2 - Distal-less homeobox 2

DMEM - Dulbecco's modified eagle's medium

DMSO - Dimethylsulfoxide

DNA - Deoxyribonucleic acid

dNTP - Deoxynucleotide

dox - Doxycycline

DSC - Dermal stem cells

E - Embryonic day

E. coli – Escheria coli

EDN3 - Endothelin 3

EM - Electron microscopy

EMT - Epithelial to mesenchymal transition

endo - endogenous

ENDRB - Endothelin receptor type B

ERK - Extracellular signal-regulated kinases

ESC - Embryonic stem cell

FACS - Fluorescence-activated cell sorting

FCS - Fetal calf serum

FOXD3 - Forkhead box D3

FOXO – Forkhead box protein O

G2/M - G2 phase/mitosis phase

G418 - Geneticin

Abbreviations

GATA4 - Globin transcription factor binding protein 4

GFP - Green fluorescent protein

GO - Gene Ontology

GSK-3 - Glycogen synthase kinase-3

h - Hour

H3K27 - Histone 3 lysine 27

H3K27me3 - H3K27 trimethylation

H3K4 - Histone 3 lysine 4

H3K9/K14 - Histone 3 lysine 9/lysine 14

HaCat - Human adult low calcium high temperature keratinocytes

HAND2 - Heart and neural crest derivatives expressed transcript 2

hESC - Human embryonic stem cell

HMG - High mobility group

HNF1 α - HNF1 homeobox A

H-RAS - Harvey rat sarcoma

HRP - Horse radish peroxidase

IFN- γ - Interferon gamma

IL-20R1 - Interleukin-20 receptor 1

IL-20R2 - Interleukin-20 receptor-2

IL-22R1 - Interleukin-22 receptor 1

IL-24 - Interleukin 24

IL-6 - Interleukin 6

IPA - Ingenuity pathway analysis

iPSC - Induced pluripotent stem cell

IRES - Internal ribosomal entry site

Abbreviations

ITGA6 - Integrin alpha 6

JAK – Janus kinase

JMJD-3.1 - JuMonJi (transcription factor) domain protein

JNK - c-Jun N-terminale kinase

KRT10 - Keratin 10

KRT14 - Keratin 14

K4 - Lysine 4

KEGG - Kyoto Encyclopedia of Genes and Genomes

K-RAS - Kirsten rat sarcoma

LB - Lysogeny broth

LEF1 - Lymphoid enhancer-binding factor-1

LOH - Loss of heterogeneity

LRR - Log R ratio

m - Milli

mAb - Monoclonal antibody

MAC1 - Macrophage-1 antigen

MAFA - Maf musculoaponeurotic fibrosarcoma oncogene family, protein A

MAPK - Mitogen-activated protein kinases

MC1R - Melanocortin 1 receptor

MCDB - Specific melanocyte medium

mda-7 - Melanoma differentiation associated gene-7

MEF2C - Myocyte enhancer factor 2C

MITF - Microphthalmia-associated transcription factor

MSA - Migration staging area

MSH – Melanocyte-stimulating hormone

Abbreviations

MT-MET-4 - Melanocyte transdifferentiated MET-4

MYOD1 – Myogenic differentiation 1

MYT1L - Myelin transcription factor 1-like

NC - Neural crest

NCC - Neural crest cells

NGN - Neurogenin

NGN2 - Neurogenin 2

NHM - Normal human melanocytes

N-RAS - Neuroblastoma rat sarcoma

NT - Neural tube

OCA1 - Oculocutaneous albinism type 1

p - p-value (significance level)

p38 - p38 MAP kinase

P53 - Tumor protein p53

PAX - Paired box

PDX1 - Pancreatic and duodenal homeobox 1

Pen/Strep – Penicillin/streptomycin

PGK - Phosphoglycerate kinase

PI3K - Phosphatidylinositol 3-kinase

PKR - Protein kinase RNA-activated

PMEL - Premelanosome protein

PuroR - Puromycin resistance

PVDF - Polyvinylidene difluoride

RAS - Rat sarcoma

RB - Retinoblastom

Abbreviations

RFP - Red fluorescent protein

RNA - Ribonucleic acid

ROS - Reactive oxygen species

rtTA - Reverse tetracycline-controlled transactivator

S phase - Synthesis phase

SCC - Squamous cell carcinoma

SCF - Stem cell factor

SDS-PAGE - Sodium dodecyl sulfate

SNP - Single nucleotide polymorphism

SOX10 - Sex determining region Y-box 10

SOX9 - Sex retermining region Y-box 9

STAT - Signal transducers and activators of transcription

TBS - Tris-buffered saline

TBX5 - T-Box 5

TCF - T-cell factor

TNF- α - Tumor necrosis factor alpha

TPA - 12-tetradecanoylphorbol 13-acetate

TRAIL - Tumor necrosis factor-related apoptosis-inducing ligand

TRE - Tetracycline responsive element

TYR - Tyrosinase

TYRP1 - Tyrosinase-related protein 1

US - United states of America

UV - Ultra violet

V - Volt

WNT - Wingless/Integrated

XIII. Acknowledgements

I would like to thank several people for their personal support and advice during the last years which promoted me to finish this work and to achieve more than I have ever thought.

First, I want to thank **Prof. Dr. Jochen Utikal** for giving me the opportunity to work on this project at the DKFZ. Additionally, he created a great work environment and took care of all administrative and organisational issues. Further, I want to thank my supervisor **Dr. Daniel Novak** for his support especially in the first few months, which helped me get settled in the lab and get started with my project. Also during the whole time of my PhD Daniel was always there to listen to my scientific challenges and giving advice. He proofread all of my abstracts, protocols and my entire thesis with great caution and gave me constructive feedback, which aided me to improve my work.

I would like to give thanks to **Prof. Dr. Viktor Umansky** for being my first examiner and one of my TAC members, for sharing his scientific knowledge and giving supportive advice to further progress. To my additional two TAC members, **Dr. Martin Sprick** and **Prof. Dr. Wiebke Ludwig-Peitsch**, I want to say thank you for their advice and always being present for answering questions and giving support besides during the TAC meetings. Thanks to **Prof. Dr. Thomas Holstein** and **Prof. Dr. Peter Angel** for being in my examination committee.

I want to thank all my lab colleagues who accompanied me during the last three years. Thank you very much for creating a nice working atmosphere, for your support and advice. Thanks to **Pan** and **Jenny** for organizing and managing the lab so well. I want to give thanks to **Mathias** and **Nathalie** for their scientific advice, help with the animal work and for being great colleagues. Especially I want to thank **Kathrin** and **Maïke** for not only being the best office mates one could imagine but also becoming good friends. Thanks for the fun time, the relaxed glass of wine and the constructive words during harsh times. I want to thank my girls **Krishna**, **Huizi**, **Frederique** and **Jing** for the awesome international cooking nights, for the distraction and relaxed evenings. I want to thank **Laura** and **Linda** for the nice time and the good conversations. Thank you all for being a friend, each on her own special way.

I want to thank my boyfriend's parents **Marliese** and **Bernd** for welcoming me in their house, for every invitation to lunch or dinner and for spontaneous ironing sessions. I want to thank his whole family for accepting me in their circle and for backing me up.

To **my parents** I want to express special gratitude for their endless support, for always being there and giving me strength. They gave me roots and wings, which offered me the opportunity to become who I am and to achieve as much as I did. No matter which decision I took and I will take, I now they will always be on my side and do everything for me that is humanly possible. Without them, I would have never reached so far. I also thank my brother **Felix** especially for his technical support with which device ever. His effort saved me many nerves.

Finally, I want to thank **Daniel** for being on my side for the last 9 years and for his unconditional love. He decided to stay with me and go through good and tough times together, no matter what. I could always count on him supporting and encouraging me. His presence gave me strength, lifted me up and made me outgrow myself.